Barley 14-3-3 Biology

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Barley 14-3-3 Biology

Peter Jonathan Schoonheim

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Barley 14-3-3 Biology

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ten overstaan van de promotiecommissie
van de faculteit der Aard- en Levenswetenschappen
op donderdag 8 februari 2007 om 13.45 uur
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De Boelelaan 1105

door

Peter Jonathan Schoonheim

geboren te Purmerend

promotor: prof.dr. H. Lill

copromotor: dr.ir. A.H. de Boer

Scientia non habet inimicum nisi ignorantem

Science has no enemies but the ignorant

Cover:
Title written in barley seeds, arched by the Saint Louis Arch. The St. Louis arch commemorates the Lewis and Clark expedition in 1804, which explored the west of the USA.

Omslag:
De titel is geschreven in gerst zaden en omringd met de St. Louis arch. De St. Louis arch is een monument dat de Lewis en Clark expeditie van 1804 symboliseerd. Deze expeditie was een van de eerste verkenningen van het westen van Amerika.

Contents

Chapter 1	General introduction	**9**
Chapter 2	14-3-3 adapter proteins are intermediates in ABA signal transduction during barley seed germination	**21**
Chapter 3	Barley 14-3-3 proteins and GA signal transduction	**51**
Chapter 4	Characterization of the barley Two Pore Channel 1 (HvTPC1)	**65**
Chapter 5	14-3-3 affinity purification in barley embryos	**79**
Chapter 6	A comprehensive analysis of the 14-3-3 interactome in barley leaves using a complementary proteomics and two-hybrid approach	**93**
References		**118**
Summary		**129**
Samenvatting		**133**
Dankwoord		**137**
Curriculum vitae		**140**

Chapter 1

General introduction and outline of thesis

Although plants do not have a central nervous system, they are capable of quickly adapting to changing environments. These adaptations are controlled by cascades of molecular networks that re-establish homeostasis (Vinocur and Altman, 2005). Signal transduction pathways can mediate signals in a time frame of seconds, like for example the signaling cascades of the Venus flytrap (*Dionaea muscipula* E.) and the Sensitive plants (*Mimosa pudica* L.). Both plants respond to touch and subsequently close their leaves in 0.5 or 2 seconds, respectively. The Venus flytrap does this to catch prey whereas the Sensitive plant will do this to protect itself against predators (Barneby, 1992). Similar to the nervous system in humans, these plants also use depolarization of membrane potentials to transduce signals (Hodick and Sievers, 1988). Another similarity with human cells is that many signal transduction pathways that are responsible for adaptations to environmental changes, the longer term signaling cascades, are controlled by hormones. Hormones are small chemical compounds that are produced in low concentrations and are synthesized at a location different from the cell where the chemical is biologically active. Hormones are in most cases transported to the locations of reception and subsequently induce a signal transduction pathway, although some hormones in plants are also biologically active at the site of biosynthesis.

Plant Hormones

Darwin was one of the first scientists who observed a hormonal regulated phenomenon called phototropism. He reported that when the tip of a coleoptile was exposed to light, a chemical messenger was produced and transported to other parts of the plant where this messenger induced an asymmetrical growth response which resulted in bending of the stem towards light (Darwin, 1880). A while later, this observation was confirmed and resulted in the isolation of the first plant hormone viz. auxin (Went, 1928; van Overbeek, 1936). Up to date, seven main groups of plant hormones have been identified viz. auxin, cytokinins, jasmonic acid, gibberellin, abscisic acid, ethylene, and brassinosteroids. Hormone signal transduction pathways are complex and in many cases are influenced by each others activities (cross-talk). Due to the complexity of the individual hormones' signal transduction pathways, scientists have been mainly focused on one or two hormone pathways. In the research described in this thesis, the focus will lie on the two antagonistic plant hormones abscisic acid and gibberellins in their regulation of barley (*Hordeum vulgare*) seed germination and plant development.

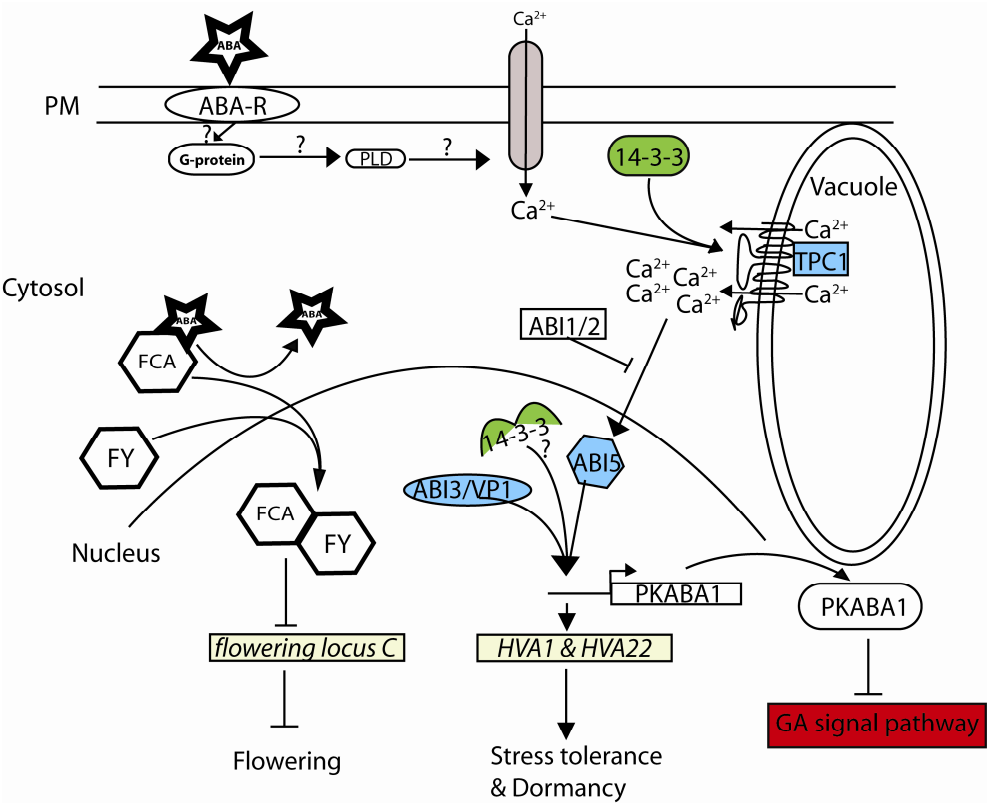

Figure 1. Cartoon of ABA perception and succeeding signal mediators

Abscisic acid signal transduction

The plant hormone abscisic acid was first identified as abscission-accelerating substance called abscisin II (Liu and Carns, 1961). Subsequently, the same molecule was isolated from sycamore leaves and induced seed dormancy; thus it was named dormin (Cornforth et al., 1966). To avoid confusion, in 1968 the molecule was renamed into abscisic acid (ABA) (Addicott et al., 1968). Since then it has become clear that ABA has a function in a wide range of physiological processes over different stages of development (Finkelstein et al., 2002). Besides these functions in developmental processes, ABA reprograms cells in order to protect the plant to environmental stress conditions, notably cold, drought and salinity (Verslues and Zhu, 2005). During seed maturation ABA establishes dormancy, prepares the seed for desiccation and controls synthesis of seed storage reserves (Finkelstein et al., 2002). Furthermore, it inhibits germination of the mature embryo and controls the transition from vegetative to reproductive growth (Rohde et al., 2000).

Forward genetic screens that have used the ABA inhibitory effects on seed germination as screening assay resulted in a wide variety of mutant plants that are either insensitive or hypersensitive to exogenously applied ABA. The identification of the responsible genetic mutations of these so called "*abi, abh, and era*" mutants have resulted in both positive and negative regulators of the ABA signal transduction pathway, ranging from transcription factors to mRNA cap binding proteins (Koornneef et al., 1984; Finkelstein, 1994; Leung et al., 1994; Allen et al., 1999; Hugouvieux et al., 2001). Intriguingly, during the last three decades scientists have been searching for the receptors that are responsible for the perception of the hormone. In the last year, both an ABA and a GA receptor have been identified (Ueguchi-Tanaka et al., 2005; Razem et al., 2006). The ABA receptor; the *FCA* gene, encodes an RNA binding protein that regulates the expression levels of a downstream gene viz. *Flowering Locus C (FLC)*. When ABA binds to FCA, ABA abolishes the binding of FCA to another protein called FY. When the FCA/FY complex is formed in the absence of ABA, it inhibits the expression of the *FLC*. Since FLC is a repressor of flowering time the FCA/FY indirectly promotes flowering (Figure 1). Thus ABA delays flowering time in the plant since it abolishes the FCA/FY complex. Surprisingly, *FCA* loss-of-function mutants do not show any impaired seed dormancy and stomatal closure, which both are classical ABA responses (Razem et al., 2006). This suggests that plants have more than one ABA receptor regulating different pathways (Figure 1). This hypothesis fits with previous findings where it was shown that the ABA receptor is located at the plasma membrane (Gilroy and Jones, 1994). Succeeding ABA perception, many signal mediators have been identified by using forward and reversed genetic approaches. Although these proteins have been shown to play an important role in the ABA pathways, for many of them it is still puzzling how and where they fit into the pathway. The most pleiotropic ABA insensitive mutants characterized up to date are the *abi1-1* and *abi2-1* mutants. These plants show altered stomatal regulation, ABA resistant seedling growth, seed dormancy, and sensitivity to exogenously applied ABA during seedling growth and seed germination (Leung et al., 1997). Due to the pleiotropic effects of these mutants it is thought that these proteins are situated upstream of the major branching points of the ABA signal transduction pathway. Nevertheless, the ABA signal transduction pathway that regulates flowering time most likely uses a different ABA receptor (FCA) and therefore is branched off before the ABI1 and ABI2 proteins. Affirmative to this hypothesis is that the *abi1-1* and *abi2-1* mutants do not show an altered flowering time phenotype (Chandler et al., 2000). Whereas ABI1 and ABI2 play an important role in many different developmental stages, transcription factors like the ABI3, ABI4, and ABI5 proteins are mainly important in the

process of embryo development and seed germination (Koornneef et al., 1984; Giraudat et al., 1992; Finkelstein, 1994; Lopez-Molina et al., 2001).

Transcriptomics studies that are performed using ABA treated plants have identified 1354 genes of which their transcript levels are regulated by ABA (Hoth et al., 2002). One of these ABA induced transcripts, the kinase PKABA1 was identified in a screen for ABA induced protein kinases (Anderberg and Walker-Simmons, 1992). In barley aleurone cells, neither ectopic expression nor knock-down of *PKABA1* has an effect on the ABA inducibility of the ABA regulated promoter *HVA1* (Zentella et al., 2002). In contrast, PKABA1 does block the GA induced upregulation of the α-amylase promoter (amy32b) (Gomez-Cadenas et al., 1999; Zentella et al., 2002). Therefore, PKABA1 is so far the only identified suitable candidate for the switch that mediates cross-talk between the ABA and GA signal transduction pathways (Ho et al., 2003). Cross-talk between de ABA and GA pathways has been shown to occur in different plant processes like seed germination, stem elongation, and expansive growth of leaf sections (Thomas et al., 1965; Chrispeels and Varner, 1967; White et al., 2000; Gomez-Cadenas et al., 2001).

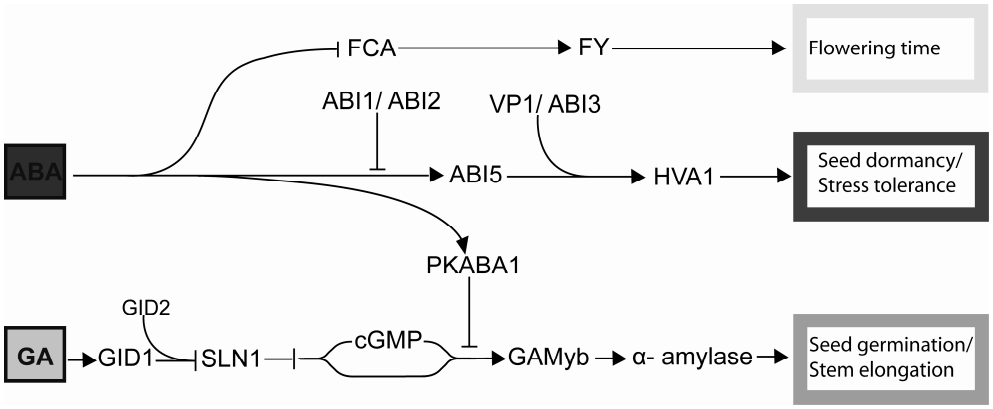

Figure 2. Diagram of the ABA and GA signal transduction pathways. Adapted from Zentella (2002) and Casaretto (2005).

Gibberellin signal transduction

Where ABA inhibits seed germination, gibberellin promotes this process and induces transcription of hydrolytic enzymes to stimulate starch breakdown. The resulting sugars are transported to the developing embryo and are used as energy supply. Gibberellins are diterpenoid compounds and are *in planta* present in many forms of which only few are biologically active (e.g. GA_1 and GA_4). Gibberellins were first identified by plant pathologists studying infected rice plants that showed elongated stems, slender leaves, and stunted roots. The fungus that infected these rice plants was shown to produce gibberellins and therefore induces a hormonal misbalance. The observed phenotype was comparable to the rice *slender* (*slr1-1*) mutant plant that was identified by performing forward genetic screens (Foster, 1977; Lanahan and Ho, 1988). The transcription factor SLR1/SLN1 is a negative regulator of the GA signal transduction pathway (Ikeda et al., 2001; Zentella et al., 2002). *Slender1* loss-of-function mutant plants show a constitutive GA response phenotype, which is independent of GA biosynthesis (Lanahan and Ho, 1988). Recently, the rice GIBBERELLIN INSENSITIVE DWARF1 (GID1) protein has been identified as the GA receptor (Ueguchi-Tanaka et al., 2005). Interestingly, GID1 interacts with SLR1 in a GA dependent manner. GID1 is a protein with unknown function and has homology with proteins of the hormone sensitive lipase family (HSL) (Ueguchi-Tanaka et al., 2005). Since in previous studies it has been shown that GA is perceived at the plasma membrane and the GID1 protein is a soluble nuclear located protein, it is hypothesized that the GA pathway also has additional receptors (Gilroy and Jones, 1994; Ueguchi-Tanaka et al., 2005).

Another protein that has been shown to interact with the SLR1, is the GIBBERELLIN INSENSITIVE DWARF2 (GID2) protein. GID2 is an F-BOX protein which is part of an ubiquitin E3 ligase complex (SCF) that is responsible for GA induced ubiquitination of proteins that are targeted for proteasome mediated protein degradation (Itoh et al., 2003; Gomi et al., 2004). Interestingly, loss-of-function GID2 rice plants show an accumulation of SLR1 proteins, indicating that SLR1 is ubiquitinated by SCF^{GID2} which triggers the 26S proteasome degradation of SLR1 (Gomi et al., 2004). The accumulated SLR1 proteins in *gid2* null mutants have been shown to be mainly phosphorylated (Sasaki et al., 2003).

Calcium as a second messenger

One of the first events that was found to be induced by GA and inhibited by ABA is the cytosolic calcium level (Gilroy and Jones, 1992). When barley aleurone cells are treated with GA, a rapid increase of the cytosolic calcium level is observed, which is subsequently responsible for the onset of α-amylase gene expression. When the same cell is treated in combination with ABA, the calcium increase, together with the induction of α-amylase gene expression is abolished (Gilroy and Jones, 1992). Increased calcium concentration functions as a second messenger and most likely results from the activation of calcium channels in the plasma membrane (Ritchie and Gilroy, 2000). Peiter et al. (2005) showed that a loss-of-function mutant of the vacuolar membrane located calcium channel *tpc1-2* has an impaired sensitivity for ABA (Peiter et al., 2005). They show that the TPC1 channel in *Arabidopsis* is responsible for the characteristic SV current in *Arabidopsis* mesophyll vacuolar membranes. Since TPC1 is a Ca^{2+}-dependent Ca^{2+} -release channel, it is hypothesized that the first Ca^{2+} signal is initiated at the plasma membrane and subsequently amplified by the vacuolar TPC1. Interestingly, van den Wijngaard et al. (2001) showed that in barley mesophyll vacuolar membranes the Slow Vacuolar (SV) current is decreased when membranes are incubated with the regulatory adapter protein Hv14-3-3B (Van den Wijngaard et al., 2001). More recently, our group also has shown that 14-3-3 protein play an important role in regulating potassium channels that regulate the influx of potassium into the cell (Van den Wijngaard et al., 2005). Interestingly, when K^+_{in} currents were measured in the presence of ABA a reduced K^+_{in} was found, which is comparable to the effect of the addition of recombinant Hv14-3-3B proteins to the vacuolar membranes.

Protein phosphorylation

Evidence that phosphorylation plays an important role in the hormone signal transduction in plants was the identification of the two serine/threonine protein phosphatase 2C (PP2C) proteins ABI1 and ABI2, which are important signal mediators in many different plant processes (Meyer et al., 1994; Leung et al., 1997; Allen et al., 1999). Although these two protein phosphatases have been identified a while ago, their target proteins still remain to be identified. Nevertheless, the importance of the ABI1 protein in the regulation of downstream target genes was shown by performing a whole genome microarray approach. Hoth et al. (2002) showed that 91.4 % of the ABA responsive genes in *Arabidopsis* are differently regulated by ABA, when plants lack the ABI1 protein (Hoth et al., 2002). In both GA and ABA pathways, the importance of phosphorylation also has been shown by the

identification of signal mediators that accumulate in a phosphorylated form, suggesting that this phosphorylation protects them from proteolytic breakdown. For example, the transcription factor ABI5 is phosphorylated in response to ABA and simultaneously shows an ABA induced stabilization of its protein (Lopez-Molina et al., 2001). The GA signal mediator SLR1, also has been shown to be more stable when phosphorylated (Gomi et al., 2004).

14-3-3 PROTEINS

Function of 14-3-3 proteins

The family of 14-3-3 proteins in recent years has come to the foreground as important adapter proteins that regulate a variety of cellular processes like apoptosis, cell division, left-right patterning, nitrogen metabolism, ABA signal transduction and GA signal transduction (Bachmann et al., 1996a; Van Hemert, 2001; Bunney et al., 2003). The 14-3-3 protein family has shown remarkable sequence conservation even throughout a variety of diverse species ranging from fungi to humans. The crystal structure of the 14-3-3 proteins has revealed that the protein forms a ≈ 60 kDa homo or heterodimer, which forms a cup shape structure (Figure 3) (Yaffe et al., 1997). Each monomer has the capability of binding one peptide and therefore a 14-3-3 dimer can bind one target protein at two different sites or bind two different targets at the same time (Braselmann and McCormick, 1995). Generally 14-3-3 proteins bind target proteins at R(S/X)X_pS/TXP or RXXX$_p$S/$_p$T/XP mode 1 and mode 2 consensus motifs, respectively (where $_p$S/$_p$T denote a phosphorylated serine or threonine). In the last decade, a stunning amount of putative 14-3-3 regulated proteins have been identified and the list is still growing almost every day. Pozuelo Rubio et al. (2004) reported over 200 phosphoproteins from HeLa cell extract that were shown to be in complex with 14-3-3 proteins. Intriguingly, these proteins regulate a variety of different cellular processes, what indicates that 14-3-3 proteins through these interactions also regulate all these processes.

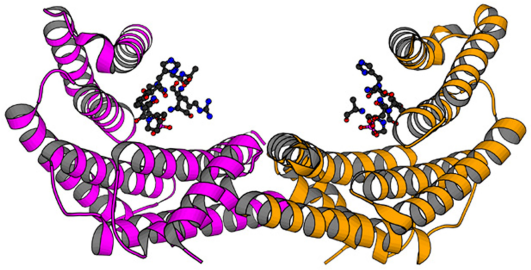

Figure 3. **Crystal structure of 14-3-3σ dimer.** Each monomer consists of nine α-helices and is depicted here in either orange or pink. Both 14-3-3 monomers are bound to a mode 1 phosphopeptide. From; (Wilker et al., 2005).

14-3-3 functions in plants

In the last decades much research has been dedicated to the identification of novel molecular targets for the 14-3-3 protein family. As discussed above, a wealth of targets have been identified ranging from transcription factors to metabolic enzymes. Surprisingly the plant 14-3-3 interacting proteins identified so far, belong mainly to metabolism related processes, e.g. glutamine synthetase, nitrate reductase, sucrose-phosphate-synthase, invertase, glyceraldehyde dehydrogenase, and many more (Bachmann et al., 1996a; Moorhead et al., 1999; Alexander and Morris, 2006). These proteins have been identified as 14-3-3 interacting proteins *in vitro* but not much is known about the *in vivo* relevance of the 14-3-3/target interaction. One of the few plant proteins of which the 14-3-3 interaction was characterized *in vivo* is nitrate reductase (NR). The 14-3-3 interaction site in the NR protein has been identified and studied extensively (Bachmann et al., 1996a; Bachmann et al., 1996b; Kanamaru et al., 1999; Lillo et al., 2003). It was shown that NR is phosphorylated in response to dark treatment and that the phosphorylation is not sufficient for NR inhibition. The phosphorylation at the C-terminally located 14-3-3 motif triggers 14-3-3 binding and subsequently results in the inhibition of NR (Lillo et al., 2003). Gain-of-function studies using a mutated NR enzyme that is not capable of binding 14-3-3 proteins showed that the abolished 14-3-3 interaction results in elevated nitrite concentrations during darkness (Lillo et al., 2003). Since the list of 14-3-3 interaction partners is long and still growing almost every day, it is necessary to study the functional relevance of the 14-3-3 interaction *in vivo*. For example by performing complementation studies using 14-3-3 interactors with mutated 14-3-3 binding motifs, as has been done for NR. Due to the diverse functions of 14-3-3 proteins it seems that classical reversed genetics approaches using knock-out mutants are not suitable. Nevertheless, some reverse genetics studies have shown that a reduction of the *Arabidopsis* 14-3-3 isoforms GF14ε and GF14μ resulted in an increased starch content in leaves (Sehnke et al., 2001). As expected, the phenotype is very complex since starch content in the leaves is dependent on many different cellular processes. Another example is the over-expression of the *Arabidopsis* GF14λ in cotton where it was shown that increased 14-3-3 protein levels confer an increased stress tolerance and also induced a “stay green” phenotype. Again, this confirms that 14-3-3 proteins regulate a wide array of processes and therefore more subtle methods should be used to study their *in vivo* functions.

14-3-3 function in plant signaling

As mentioned above, in plants 14-3-3 proteins have been mainly described as regulators of metabolism related processes. Nevertheless, one of the first identified plant 14-3-3 protein was characterized as G-Box binding factor (GBF), hence the GF14 names for *Arabidopsis* 14-3-3 proteins (Lu et al., 1992a). G-Boxes are *cis*-acting promoter elements present in many diverse genes. G-boxes were first found to regulate the light regulated ribulose-1,5-bisphosphate-caboxylase/oxygenase small unit (*rbcS*) (Giuliano et al., 1988). Therefore G-boxes were thought to be specific promoter elements that play a role in light regulated gene expression. Later, G-boxes were found to be switched on and off by a variety of different stimuli like the anoxia induced alcohol dehydrogenase (*ADH*) (McKendree et al., 1990), the UV-light regulated chalcone synthase (*CHS*) (Schulze-Lefert et al., 1989), and the abscisic acid induced *Em (Guiltinan et al., 1990)*. G-boxes located in the ABA inducible promoters from *Em, HVA1, and HVA22* genes are called ABA responsive elements (ABRE). The ABREs in combination with coupling elements (CE) located near the ABRE, are responsible for the ABA induced activation of gene expression (Shen et al., 2004). Up to date, many ABRE binding factors have been identified of which most belong to the bZIP transcription factor family (Foster et al., 1994; Menkens et al., 1995; Jakoby et al., 2002; Kim, 2006). Schultz et al. (1998) identified two 14-3-3 interactors that together with 14-3-3 proteins are present in ABRE of the promoter of the *Em* gene viz. EmBP1 and VP1 (Schultz et al., 1998). Since it was shown that the VP1 transcription factor does not bind the ABRC in the *Em* promoter itself (Suzuki et al., 1997), it was hypothesized that 14-3-3 proteins form the bridge between the EmBP1 and the VP1 proteins (Himmelbach et al., 2003). Interestingly, de Vetten et al. (1994) showed that 14-3-3 proteins themselves contain G-box elements in their own promoter, and also showed that 14-3-3 proteins are present in promoter complexes that can bind their own promoter. Recently, it was shown that indeed some of the 14-3-3 genes are ABA inducible (Chen et al., 2006), and it is shown in this thesis that 14-3-3 proteins play an important role in the ABA induction of downstream genes (Chapter 2) and thus possibly also regulate the expression of their own genes.

Critical remarks

The main question in 14-3-3 biology to date is how 14-3-3 proteins regulate so many different proteins with so many different functions at the same time? The answer to this question is not so straight-forward; most likely the following factors have to be considered. First, cellular localization most likely plays an important role. When cell extracts are prepared, a diversity of differentiated cells are homogenized and therefore an unnatural protein mixture is prepared. 14-3-3 interactions can be measured amongst these proteins but *in vivo* these interactions may not occur simply because the two proteins would never or only occasionally meet in one cell. Second, subcellular localization plays an important role as well; the cell is a well organized maze of actin filaments and microtubules. Proteins are carefully guided to one another's active site and therefore protein-protein interactions occur probably not just by chance, but are accommodated by many localization regulated events. Third, target proteins themselves need to be phosphorylated to form a high affinity binding site for 14-3-3 proteins and therefore kinases are the decision makers whether a 14-3-3 protein binds or not. Fourth, 14-3-3 proteins themselves have been shown to be phosphorylated and polyglycylated as well and this forms another regulatory step that can differentiate binding between specific targets (Lalle et al., 2006). Fifth, 14-3-3 proteins are known to form hetero- and homodimers, therefore many different combinations of 14-3-3 isoforms can be formed what may result in the perfect regulator. Unfortunately, the functional role of 14-3-3 hetero-dimerization is poorly understood. To our knowledge, so far all functional assays with 14-3-3 proteins have been performed with homo-dimers.

Outline of thesis

Since 1992, the up to then “bovine brain specific protein” of the 14-3-3 protein family, was identified in other tissues and organisms as well. It became clear that this abundant protein plays an important role in many different processes and in many different organisms. In the studies described in this thesis we have tried to answer the following questions: The barley genome has not been sequenced yet and therefore it is unknown how many 14-3-3 genes are present. Three 14-3-3 genes have been identified thus far viz. 14-3-3A, 14-3-3B, and 14-3-3C. In **chapter 2** we identified two novel 14-3-3 genes viz. 14-3-3D and 14-3-3E. We studied the 14-3-3 gene expression and protein levels of the five barley 14-3-3 isoforms in barley radicles. We discovered that the 14-3-3 genes expression and proteins levels are affected by treatment with the hormone abscisic acid. As a follow-up to this, we studied the functionality of the 14-3-3 proteins by using RNA interference. We found that each 14-3-3 isoform plays a role in the ABA induction of gene expression. Moreover, by using the yeast two-hybrid principle we showed that the barley 14-3-3 proteins interact with four of the barley ABF/AREB/ABI5 bZIP transcription factor family viz. HvABI5, HvABF1, HvABF2, and HvABF3. In **Chapter 3** we used the same method as in chapter 2 but here we studied whether 14-3-3 proteins play a role in the ABA antagonistic pathway, namely the gibberellin (GA) pathway. We studied and confirmed that the barley ortholog of the tobacco RSG bZIP transcription factor viz. HvRF2A, which plays a role in the GA biosynthetic pathway, interacts with all five barley 14-3-3 isoforms. We showed that each 14-3-3 isoform plays a role in the GA induced gene expression of the α-amylase promoter. We found that 14-3-3 proteins reduce the GA inducibility of the α-amylase promoter and therefore we studied whether the DELLA transcription factor SLN1 interacts with the 14-3-3 protein. Both GA and ABA signal transduction pathways have been shown to use Ca^{2+} as a second messenger. In a recent report it was shown that the *Arabidopsis* Two pore calcium channel (TPC1) plays an important role in the ABA regulation of seed germination. In **Chapter 4** we studied whether the barley TPC1 protein has the same function as the *Arabidopsis* ortholog. We show that 14-3-3 protein isoform A interacts with the cytosolic loop of the TPC1 protein. **Chapter 5 and Chapter 6** both describe the identification of novel putative 14-3-3 regulated proteins. We used complentary approaches, viz. 14-3-3 affinity purification and yeast two-hybrid screens to identify these novel putative 14-3-3 interactors. We isolated over 150 novel interactors and studied the AREB/ ABI5/ABF like transcription factor family in more detail.

Chapter 2

14-3-3 adapter proteins are intermediates in ABA signal transduction during barley seed germination

With: Mark P. Sinnige, Jose A. Casaretto, Helena Veiga, Tom D. Bunney, Ralph S. Quatrano and Albertus H. de Boer.

Plant J. (2007) xx: xxx-xxx

ABSTRACT

Proteins of the 14-3-3 family have well-defined functions as regulators of plant primary metabolism and ion homeostasis. However, neither their function nor action mechanism in plant hormonal signaling has been fully addressed. Here we show that abscisic acid (ABA) affects both expression and protein levels of five 14-3-3 isoforms in embryonic barley roots. Since ABA prolongs the presence of 14-3-3 proteins in the elongating radicle, we tested whether 14-3-3s are instrumental in ABA action using RNA interference. Transient co-expression of 14-3-3 RNAi constructs along with an ABA responsive promoter shows that each 14-3-3 is functional in generating an ABA response. In a yeast two-hybrid screen we identified three new 14-3-3 interactors that belong to the ABF protein family. Moreover, using a yeast two-hybrid assay we show that the transcription factor HvABI5, which binds to cis-acting elements of the ABA inducible HVA1 promoter, interacts with three of the five 14-3-3s. Our analyses identify two 14-3-3 binding motifs in HvABI5, which are essential for 14-3-3 binding and proper *in vivo* trans-activation activity of HvABI5. In line with these results, 14-3-3 silencing effectively blocks trans-activation. Our results indicate that 14-3-3 genes/proteins are not only under control of ABA, but that they control ABA action as well.

INTRODUCTION

During seed maturation ABA establishes dormancy, prepares the seed for desiccation and controls synthesis of seed storage reserves (Finkelstein et al., 2002). Furthermore, it inhibits precocious germination of the mature embryo and controls the transition from vegetative to reproductive growth (Rohde et al., 2000). Besides these functions in developmental processes, ABA rearranges the molecular composition of the cell in order to adapt to environmental stress conditions, notably cold, drought and salinity (Verslues and Zhu, 2005).

Knowledge of how ABA functions at the molecular level is still fragmentary. The receptor for ABA has remained elusive until recently (Razem et al., 2006). The nature of the newly discovered receptor is unconventional; an RNA-binding protein (FCA in *Arabidopsis*, ABAP1 in barley) that regulates flowering time. Notably, FCA protein is not required for seed germination or the stomatal response to ABA. This supports the idea that there are multiple ABA response mechanisms ('receptors') operating at the same time; e.g. membranes that change fluidity when the lipophilic ABA inserts, enzymes or ion transporters having allosteric sites for ABA binding (Rock, 2000), or binding proteins with receptor-like properties like RPK1 (Osakabe et al., 2005).

Combined genetic, molecular, biochemical and biophysical approaches have succeeded in the identification and characterization of a wide range of genes with a function in ABA signaling (Finkelstein et al., 2002). Amongst these genes are positive (e.g. OST1, RPK1 and RCN1) and negative (e.g. ABI1, ABI2, ERA1, ABH1, TPC1, ROP10 and AtRAC1) regulators of ABA signaling, as well as transcription factors belonging to the basic leucine zipper (bZIP) class proteins, designated as ABFs and AREBs (Hobo et al., 1999; Choi et al., 2000; Uno et al., 2000). Despite this wealth of signaling components, it is not clear what the physical or biochemical interactions amongst these network components are. Signaling networks often rely on the formation of dynamic protein complexes, with kinases/phosphatases determining the strength of protein-protein interaction through (de)-phosphorylation. Scaffold proteins are important elements of such complexes and one family of proteins known to fulfill an important scaffold function in all eukaryotic cells is the 14-3-3 protein family (Tzivion et al., 2001; Sehnke et al., 2002; Mackintosh, 2004; Thomas et al., 2005). Intriguingly, cells seem to have hundreds of proteins that are targeted by 14-3-3s, and many of these targets have a mutual relationship because they operate in networks, like apoptosis, cell cycle control and ion homeostasis (Van Hemert, 2001; Van den Wijngaard et al., 2005).

14-3-3 proteins are acidic regulatory proteins forming homo- and hetero-dimers. A 14-3-3 dimer forms a clamp-shape structure (Yaffe et al., 1997) that can interact with one protein at two different positions or bind two different target proteins (Braselmann and McCormick, 1995). The formation of a complex between 14-3-3 and one (or two) targets can have a range of context-dependent effects, including compartmental sequestration, conformational change, enzyme (in)activation, shielding, re-localization, and bridging between two molecules. Unlike in animal cells, where 14-3-3 proteins are major determinants of cellular signaling (Van Hemert, 2001), plant 14-3-3s are best characterized as regulators of proteins with a function in C- & N-metabolism (Huber et al., 2002) and ion homeostasis (Bunney et al., 2002; Van den Wijngaard et al., 2005).

In plants, thus far only a small number of reports link 14-3-3 to proteins with a function in signal transduction. Camoni et al., (1998) reported the 14-3-3 induced activation of a calcium-dependent protein kinase (CPK1). This kinase inhibits the calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis* through phosphorylation (Hwang et al., 2005).

Evidence for a function of 14-3-3 in the action of ABA is still fragmentary or indirect. E.g. ABA signaling in guard cells antagonizes the blue light-dependent H^+ pumping through dephosphorylation of the pump and release of 14-3-3 (Zhang et al., 2004) and in embryonic roots ABA inactivates a 14-3-3 activated inward K^+-channel (Van den Wijngaard et al., 2005). At the level of transcriptional regulation, it was reported that the ABA signaling effecter VP1 (viviparous 1), interacts with 14-3-3 in a yeast two-hybrid assay (Schultz et al., 1998). VP1 is a transcriptional activator, but it does not directly bind to the cis-acting elements (so-called ABRE's) present in promoters of ABA-inducible genes (Suzuki et al., 1997). It has been suggested that 14-3-3 proteins function as adapters between VP1 and the ABRE-binding trans-acting factors AREB/ABF/ABI5 family (Himmelbach et al., 2003). The ABF/AREB/ABI5 family is a key target of a conserved ABA signaling pathway in plants, their transcript and protein accumulation, phosphorylation state, stability, and activity are highly regulated by ABA during germination and early seedling growth (Finkelstein and Lynch, 2000b; Lopez-Molina et al., 2001; Carles et al., 2002; Kagaya et al., 2002; Casaretto and Ho, 2003; Furihata et al., 2006).

In this study we used the HvABI5- and VP1-dependent ABA inducible transcription of the ABRC3 cis-acting elements derived from the *HVA1* promoter to address the question whether and how 14-3-3 proteins act as effecters in ABA signaling during seed germination. Our results show that silencing each individual member of the barley 14-3-3 family (five genes identified) dramatically reduces the activity of ABRC3-GUS. We

provide evidence that the transcription factors HvABF1, HvABF2, HvABF3, and HvABI5 interact with 14-3-3 proteins. Moreover, we show that the threonine residues, HvABI5-T^{350}, at the very C-terminus, in a canonical 14-3-3 binding motif, is essential for interaction with HvABI5. Mutation of this HvABI5-T^{350} reduces the activity of the HvABI5 for its trans-activation of the ABRC3-GUS. We discuss the function of 14-3-3 proteins in ABA-dependent signal transduction in seed dormancy and germination.

RESULTS

Identification of two new barley 14-3-3 genes

Phylogenetic analysis, combining the three published barley 14-3-3 proteins (known as Hv14-3-3A, B, and C in the Genbank database) with 14-3-3 proteins from three other monocotyledonous plants (rice, maize and wheat) showed that the proteins can be divided in five evolutionary clusters (Figure 1B). Two of the five clusters contain a barley ortholog, indicating that three barley genes remained to be identified. Therefore, a PCR based screen was performed to identify these other barley 14-3-3 isoforms. Our search, which was based on the homology with other plant 14-3-3 proteins, yielded two novel barley 14-3-3 genes (Figure 1A). These two new 14-3-3 proteins, named Hv14-3-3D and Hv14-3-3E, show 75% similarity between each other, and more than 70% similarity with the other three already known 14-3-3 isoforms (amino acid level). A noticeable difference between Hv14-3-3E and the other four proteins is found in the loop between helix 2 and 3, where Hv14-3-3E contains four extra amino acids (Figure 1A). This extension of loop 1 is also present in the wheat and rice homologue and is not found in any of the animal 14-3-3 proteins. Despite our extensive search, no sixth barley 14-3-3 could be identified so far.

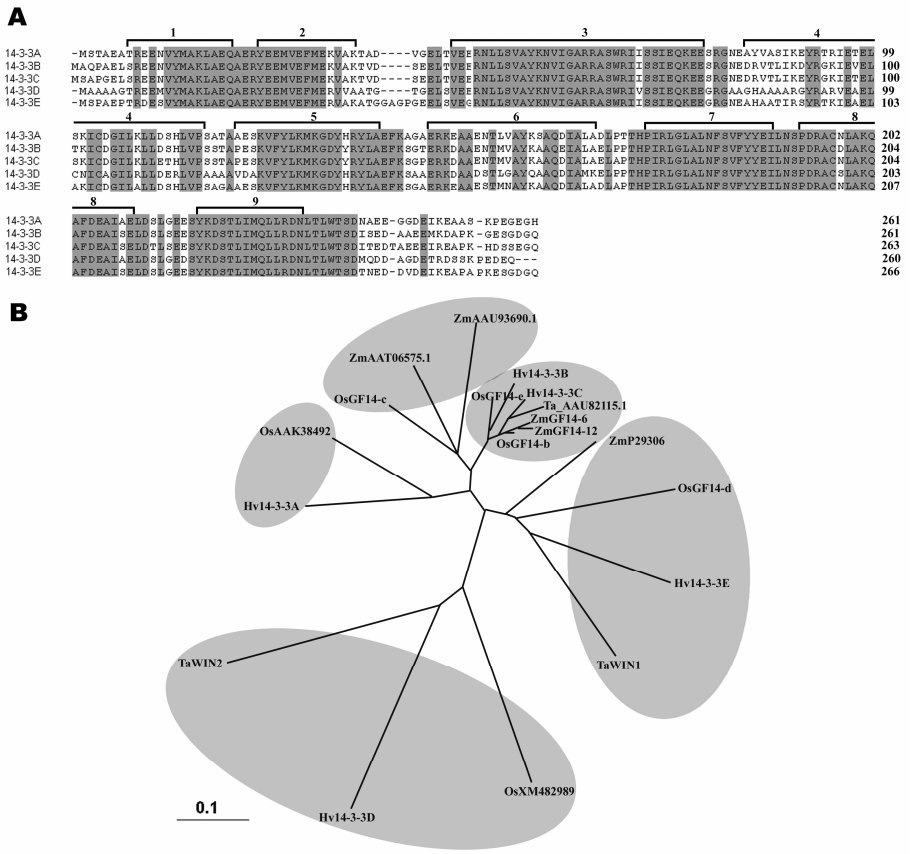

Figure 1. Sequence analysis of barley 14-3-3 proteins.

(A) Alignment of the five barley 14-3-3 isoforms. Predicted α-helixes are marked 1 to 9. **(B)** Phylogenetic tree of 14-3-3 proteins from monocot plants rice, maize, wheat and barley, shows that the monocot plant 14-3-3 protein families can be divided in 5 clusters.

ABA controls expression and protein levels of Hv14-3-3 in the embryonic root of germinating barley seeds

Since the embryonic root (radicle) is a good model system to study ABA action (Finkelstein and Lynch, 2000a; Van den Wijngaard et al., 2005) the 14-3-3 expression patterns were analyzed by Q-PCR and corresponding protein levels by means of isoform-specific antibodies. Radicles were isolated from imbibed seeds 20 hours after imbibition, just prior to emergence from the coleorhiza. Growth analysis of ABA-treated and control roots shows the viability of the isolated roots and their responsiveness to ABA (Figure 2F).

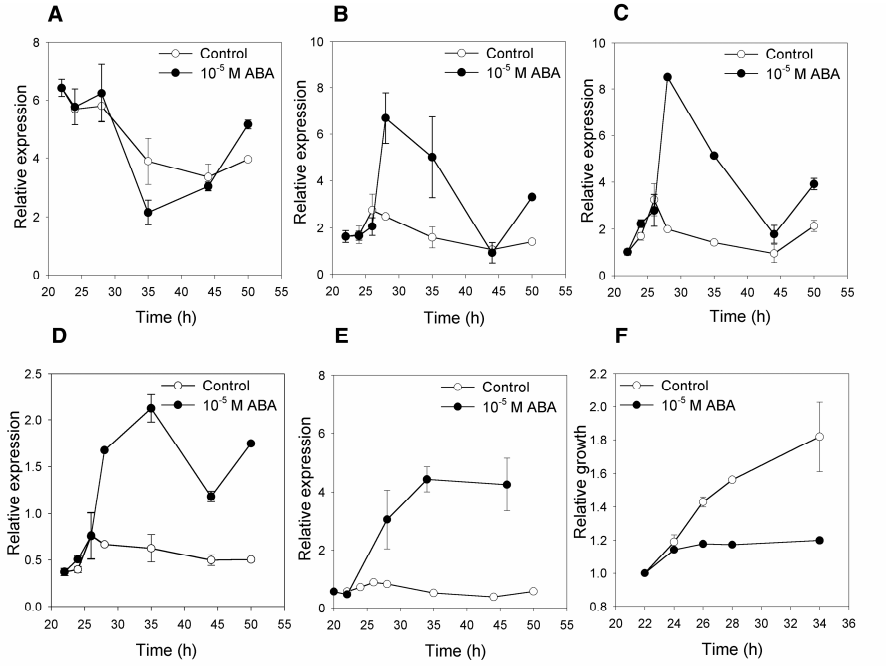

Figure 2. Growth and expression profiling of 14-3-3 genes in untreated and ABA treated barley radicles.

(A-E) ABA affects the expression levels of four of the five 14-3-3 isoforms. Figures A to E correspond to isoforms A,B,C,D, and E respectively. Relative expression levels of five 14-3-3 isoforms in radicles were followed in untreated radicles (○) and ABA treated (●; 10 µM). Time is indicated as hours after imbibition (h). Expression levels are normalized on the housekeeping gene; *Actin* (n=3) ± SE. The stability of the expression of the *Actin* gene in growing radicles was tested using a second housekeeping gene viz. *GAPDH* (data not shown). **(F)** Relative growth (FW increase) of isolated radicles is followed in time (normalized to fresh weight of starting material). Growth of untreated (○) radicles and ABA (●; 10 µM) treated radicles are shown (n=2) ± SE. Radicles were isolated 20 hours after imbibition and treatments started at 22 hours after imbibition.

The expression data shows that four of the five *14-3-3* isoforms respond to ABA treatment (Figure 2A-E). The expression levels of the *Hv14-3-3A* isoform are not differently affected by the ABA treatment compared to control radicles, whereas ABA induces a transient up-regulation of the *Hv14-3-3B* and *Hv14-3-3C* isoforms, peaking at six hours after start of treatment (Figure 2A-C). ABA induces a strong and sustained up-regulation (six to eight-fold) of the two newly identified *Hv14-3-3D* and *Hv14-3-3E* genes (Figure 2D and E).

Just like gene expression, also protein levels of four of the five 14-3-3 isoforms are affected by ABA. The Hv14-3-3A isoform is post-translationally modified and is cleaved into a 28 kDa protein in untreated radicles (Figure 3). This truncation of Hv14-3-3A has been shown before in germinating barley embryos (Testerink et al., 2002). However, here we demonstrate that treatment with ABA prevents the proteolytic cleavage of Hv14-3-3A (Figure 3). Protein levels of Hv14-3-3B are not affected by ABA. Hv14-3-3C protein levels in untreated radicles show a transient increase around 28 hours after imbibition and the protein has disappeared 46 hours after imbibition. In ABA treated radicles Hv14-3-3C shows the same increase after 28 hours after imbibition, but now this level is maintained even after 46 hours (Figure 3). Proteins of isoforms D and E disappear in untreated radicles within 1 to 1½ day after imbibition, but ABA treatment prevents this reduction (Figure 3). Taken together, these results show that both *14-3-3* gene expression and 14-3-3 protein levels are controlled by ABA in an isoform-specific manner. In view of these responses to ABA it is plausible that 14-3-3 proteins are instrumental in the action of ABA to maintain seed dormancy.

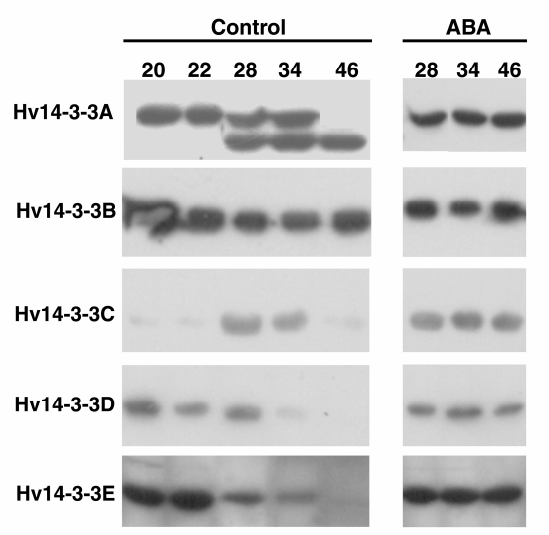

Figure 3. ABA stabilizes 14-3-3 proteins in isolated radicles.

Western-blots of total protein extracts (15 µg) of untreated- and ABA (10 µM) treated radicles. Time is shown in hours after imbibition. Radicles were isolated 20 hours after imbibition and treatments started at 22 hours after imbibition. Three independent experiments were performed and representative results are shown. Western-blots, prior to hybridization with primary antibody, were stained by Ponceau S (BIO-RAD) to check for equal loading.

14-3-3 proteins are regulators of the ABA inducible ABRC3 derived from the HVA1 promoter

We next investigated whether some, or all, of these 14-3-3 isoforms have a function in the signal transduction cascade of ABA that results in gene activation. The presence of 14-3-3 proteins in G-box binding complexes has been shown before (de Vetten et al., 1992; Lu et al., 1992b; Schultz et al., 1998) and supports our hypothesis that 14-3-3 proteins may be important for ABA signal transduction. However, the molecular mode of action of 14-3-3 proteins has not been shown in these protein-DNA complexes. To address this question, we studied the ABA signal transduction pathway by biolistic transformation of embryo-less half seeds and silencing the *14-3-3* isoforms using RNA interference. To follow ABA signaling, the promoter activity of a well-characterized ABA inducible gene, *HVA1*, was measured as output of the ABA signal transduction pathway. Aleurone layers of barley seeds have been shown to be a good model for biolistic transformation and RNA interference studies (Zentella et al., 2002). To show that the ABA responsiveness of *HVA1*

in the aleurone layer of embryo-less half seeds corresponds to that in our radicle system, the expression levels of the *HVA1* were followed in radicles by Q-PCR. As shown in Figure 1 of the supplementary material, *HVA1* responds quickly and is highly up-regulated in response to ABA in barley radicles.

The ABRC3-GUS reporter construct that contains the ABA responsive *cis*-acting elements from the *HVA1* promoter that are necessary and sufficient for ABA induction is fused to GUS (Shen, 1996). The ABRC3-GUS fusion is used to measure ABA induction of gene expression *in vivo* (Casaretto and Ho, 2003). Silencing of the *14-3-3* isoforms was performed using isoform-specific RNA interference constructs designed against the 3' UTR sequence of the *14-3-3* genes (Supplementary material; Figure 3). As control, an 'empty' plasmid was co-bombarded that contained the UBI1 promoter.

The isoform-specific 14-3-3 RNAi constructs were individually co-bombarded with the ABRC3-GUS construct. In order to minimize variations among particle bombardments, luciferase under control of the ubiquitin promoter (UBI-Luc) is used as internal control. Since 14-3-3 proteins have multiple targets and are capable of inhibiting enzymes, we first ruled out the possibility that 14-3-3 RNAi has an effect on the luciferase enzyme activity by performing the following experiment: The UBI-Luc and UBI-GUS construct were co-bombarded with and without 14-3-3RNAi constructs. In this way the luciferase activity could be normalized on the GUS activity. The 14-3-3 RNAi constructs did not have an effect on the luciferase activity (data not shown).

Figure 3 of the supplementary material shows that the ABRC3-GUS is upregulated by ABA. Bombardment of the effector construct targeted to *Hv14-3-3A*, resulted in a reduction of 40% of the ABRC3-GUS expression in the presence of ABA. Silencing of the other four *14-3-3* isoforms induced a reduction of ABRC3-GUS activity in the presence of ABA of at least 70%. 14-3-3E-RNAi showed the strongest reduction of the ABRC3-GUS activity; up to 90%. ABRC3-GUS activities of non-treated seeds also show a reduced activity in response to the 14-3-3 RNAi construct. We hypothesize that this is due to the endogenous ABA that triggers the ABA signal transduction in the aleurone cells, which is reduced due to the lack of 14-3-3 proteins. The same effect has been observed when these kinds of experiments have been performed with the transcription factor HvABI5 (data not shown).

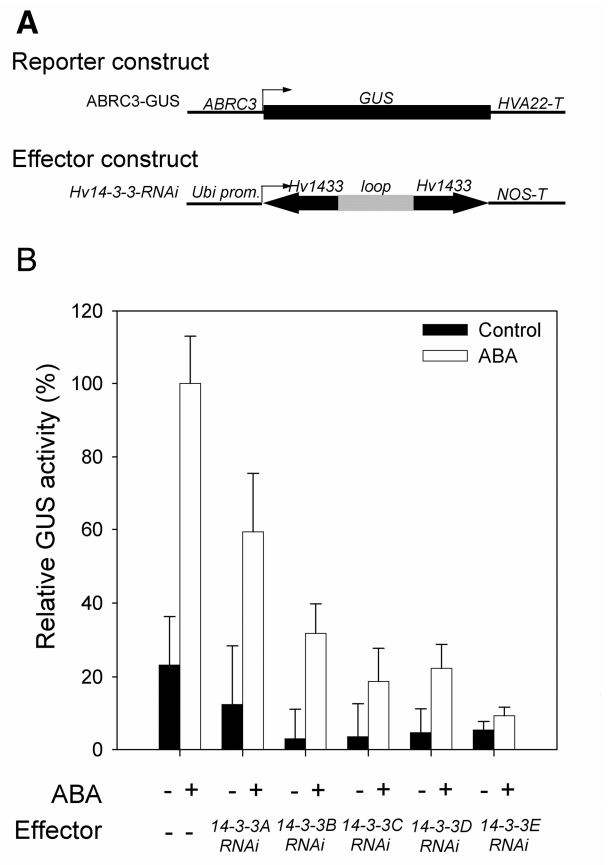

Figure 4. Transient transformation of barley aleurone cells with 14-3-3 RNAi-generating constructs suppresses the ABA induction of the ABRC3-GUS.

(A) Schemes of reporter and effector constructs. RNA interference constructs of the *Hv14-3-3* genes were targeted to the 3' UTR region of each gene. **(B)** ABA responsiveness of the ABRC3-GUS was followed in the aleurone layer of embryo-less barley half seeds by biolistic transformation of the ABRC3-GUS fusion. Untreated (solid bars) and ABA (20 µM) treated (white bars) barley seeds are shown (n=4) ±SE.

Specificity of RNA interference

Because silencing of each of the five *14-3-3* isoforms showed in all cases a reduction of the ABRC3-GUS activity, it raised the question whether there was cross silencing of the other not targeted genes. As shown in Figure 3 of the supplementary material, the sequences used for the inverted repeat constructs were chosen such that each RNAi construct would silence only the *14-3-3* where it is derived from and not the others. To test the isoform specificity of the RNAi constructs, we prepared a fusion of the *Hv14-3-3E* gene with a *GUS* reporter gene. The 3' end of the *Hv14-3-3E* gene (part of the coding sequence and the 3' untranslated region, which includes the RNAi target sequence) was fused to the 3' end of the GUS gene, between its stop codon and the NOS-terminator (see Figure 5A). This GUS-14-3-3E construct was co-bombarded with the isoform-specific RNAi constructs. Breakdown of GUS-14-3-3E RNA by the RNA interference machinery will result in a decrease of GUS proteins and thus can be measured in the GUS activity assay.

Figure 5B shows that the RNAi construct derived from *Hv14-3-3E*, silences the GUS-14-3-3E with an efficiency of approximately 60%. Moreover, the RNAi constructs directed against the other *14-3-3* isoforms do not reduce the GUS activity and thus indicates that the RNAi constructs are specific. This provides evidence that every individual member of the barley 14-3-3 protein family has a function in ABA induced activation of the ABRC3 promoter elements in barley aleurone cells.

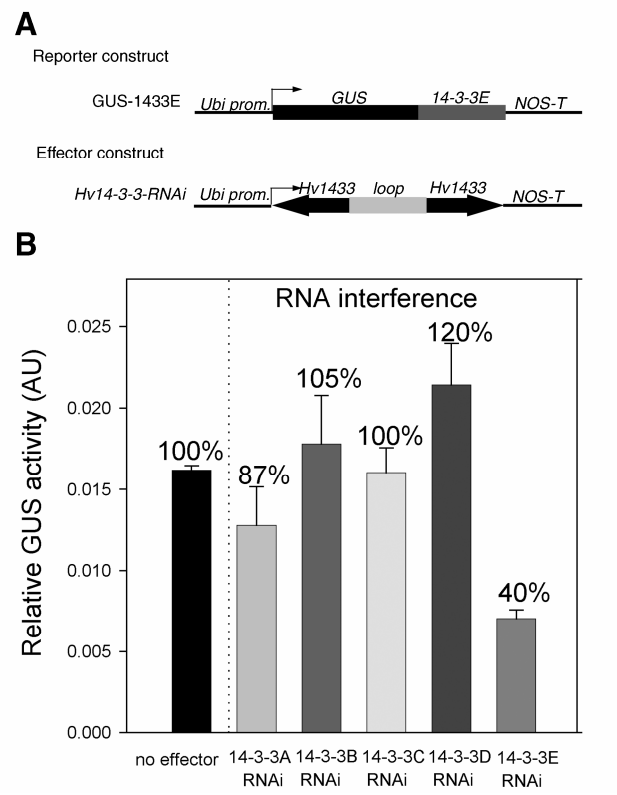

Figure 5. Silencing constructs are specific and show no signs of cross-silencing.

(A) Scheme of the reporter and effector constructs. Part of the *14-3-3E* cDNA sequence was fused to the 3' end of the GUS reporter gene. *14-3-3* isoform specific RNA interference constructs were used. **(B)** Relative GUS activities of the bombardments are shown. The control bombardment was set to 100% showing that the 14-3-3E RNAi construct is the only construct that reduces the GUS activity of the GUS/14-3-3E fusion.

Yeast two-hybrid screen with 14-3-3 proteins as bait to identify molecular targets in the ABA signal transduction pathway

A main hallmark of 14-3-3 action is that 14-3-3 proteins act as master regulators in specific signaling networks, like the cell cycle, apoptosis and hormonal signaling (Van Hemert, 2001). Since the ABA signal transduction pathway forms a complex network with many signal mediators (Rock, 2000), it is not unlikely that there are multiple 14-3-3 targets within this network. HvABI5 and VP1 are two transcription factors that have been shown to be important mediators of the ABA signal in barley aleurone cells (Casaretto and Ho, 2003) and one of these (ZmVP1) was shown to interact with rice 14-3-3 proteins GF14a, GF14b and GF14c in a yeast two-hybrid assay (Schultz et al., 1998). In a yeast two-hybrid screen, using a cDNA library constructed from RNA derived from seven days old barley leaves and all five individual barley 14-3-3 isoforms as bait, we identified one hundred and thirty-two new 14-3-3 target proteins (Chapter 6). Amongst these targets were three AREB/ABF/ABI5 like proteins (Figure 6A and B). We named the newly identified AREB/ABF/ABI5 proteins after their *Arabidopsis* homologues viz. HvABF1, HvABF2, and HvABF3 (Figure 2A; supplementary material).

In view of this result, we addressed the question whether the seed-specific ABF-homologue, HvABI5, might interact with 14-3-3 proteins as well. The HvABI5 protein has two putative 14-3-3 interaction motifs located around T^{160} (REIPT^{160}AP) and T^{350} (RRTLT^{350}GP), whereat the latter motif is conserved in most of the ABF proteins (Figure 2B; supplementary material). A yeast two-hybrid assay using the five 14-3-3 isoforms as prey and HvABI5 as bait showed that HvABI5 interacts with three of the five 14-3-3 isoforms: Hv14-3-3C, Hv14-3-3D, and Hv14-3-3E (Figure 6C and D).

The *in vivo* interactions of the 14-3-3 proteins with the AREB/ABF/ABI5 like proteins were confirmed *in vitro* by performing far-western analysis using recombinant proteins (Figure 6E). This interaction between 14-3-3 proteins and HvABI5 might explain why 14-3-3 silencing prevents activation of the ABRC3-GUS in response to ABA (Figure 4). Therefore, we decided to focus on the role of HvABI5 / 14-3-3 interaction *in vivo*, by mutating the 14-3-3 binding site in the HvABI5 protein and follow its activity in the trans-activation of the ABRC3-GUS.

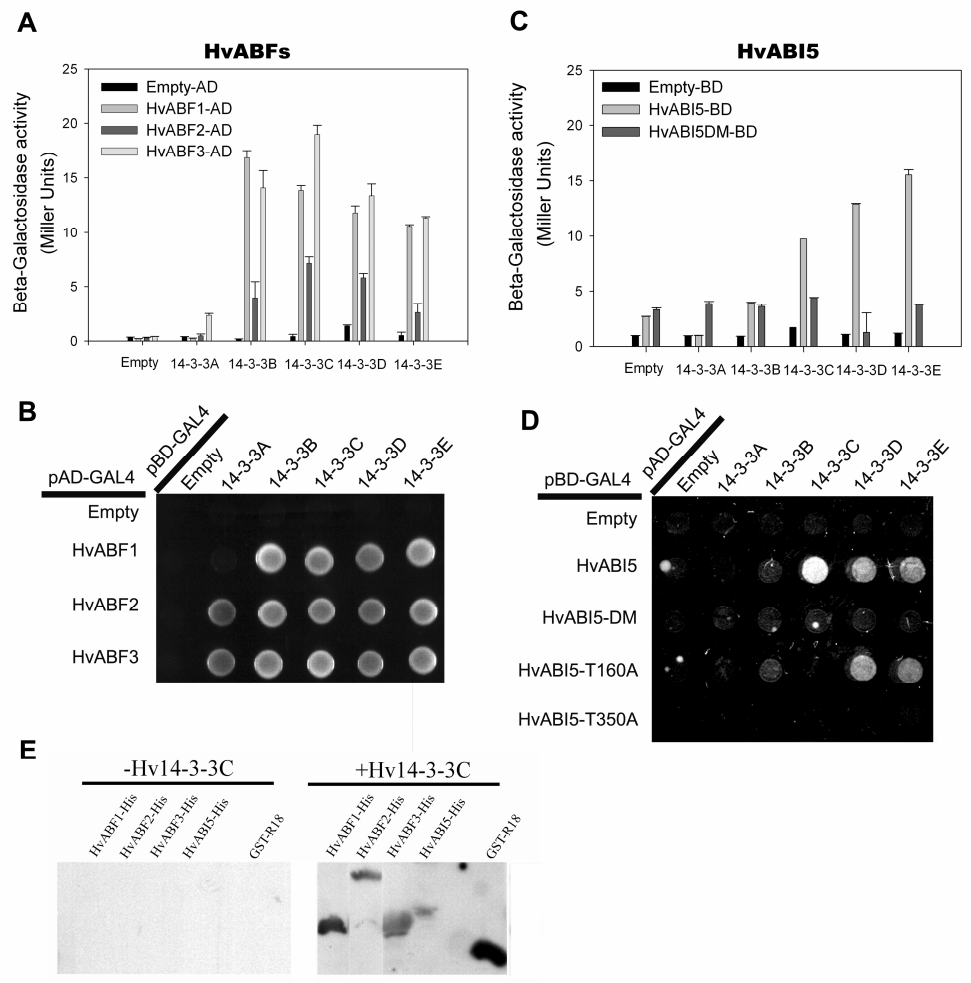

Figure 6. 14-3-3 proteins interact with four AREB/ABF/ABI5 like proteins in yeast two-hybrid assays and in Far-Western blotting.

Yeast two-hybrid assays confirm the interaction between barley 14-3-3 proteins and four AREB/ABF/ABI5 like proteins. **(A & C)** Interactions are shown quantitatively by measuring β-galactosidase activity (n=3) ±SE. **(B & D)** The interactions of the three HvABF and the HvABI5 proteins containing the mutant forms of HvABI5 were measured qualitatively. Full-grown yeast cultures were spotted on selective media (SD -LWHA) and were grown for an additional 3-5 days. **(E)** Validation of the yeast two-hybrid assays by performing Far-Western analysis using recombinant biotinylated Hv14-3-3C in the overlay buffer and recombinant histidine tagged HvABFs/HvABI5 as target proteins on blot, GST-R18 peptide that is known to bind 14-3-3 with very high affinity, proves that the far western assay is functional.

To identify the 14-3-3 binding site in the HvABI5 protein, we introduced point mutations in two putative 14-3-3 interaction motifs. The T^{160} and T^{350} amino acids of HvABI5 were substituted for an alanine amino acid resulting in three mutant forms of HvABI5: HvABI5T^{160}A, HvABI5T^{350}A and the double mutant HvABI5T^{160}A + T^{350}A (HvABI5dm). These mutants were tested in the yeast two-hybrid assay for interaction with all five 14-3-3 isoforms. Mutation of both putative interaction motifs (double mutant) in HvABI5 resulted in a complete loss of interaction with Hv14-3-3C, Hv14-3-3D and E (Figure 6D). Assays with the individually mutated threonine residues showed an interesting isoform-specific interaction with the HvABI5 protein. It was found that the T^{160} mutation (HvABI5T^{160}A) disrupts only the interaction with the Hv14-3-3C isoform, whereas isoform Hv14-3-3D and E were still capable of binding to the HvABI5T^{160}A protein. In contrast to the T^{160} mutation, the T^{350} mutation (HvABI5T^{350}A) disrupted the interaction with all three interactors, Hv14-3-3C, D & E (Figure 6D). From this we conclude that the canonical 'RRTLT^{350}GP' motif is essential for interaction between HvABI5 and Hv14-3-3C, D and E. However, both 14-3-3 binding sites, viz. REIPT^{160}AP and RRTLT^{350}GP, are necessary for interaction with the Hv14-3-3C protein.

Trans-activation of ABRC3-GUS by ZmVP1 and HvABI5 is dependent upon the 14-3-3 protein binding site of the HvABI5 protein

In the next step, we tested whether the intact C-terminal 14-3-3 interaction motif (around T^{350}) of the HvABI5 protein is essential for the *in vivo* action of HvABI5. To do so, we performed transient trans-activation experiments to analyze the effect of amino acid substitutions and deletions on its trans-activation activity. Ectopic expression of HvABI5 alone, has no or very little effect on the activity of the ABRC3-GUS (Casaretto and Ho, 2003). However, when HvABI5 is co-bombarded together with ZmVP1, the ABRC3-GUS is trans-activated and ABA treatment does not have an additional effect on the activity of the ABRC3-GUS (Figure 7). We tested three mutant forms of HvABI5 for their ability to trans-activate the ABRC3-GUS together with ZmVP1; T^{350}A, T^{350}D and C4Del, where the last 4 C-terminal amino acids (including T^{350}) are missing in the C4Del mutant protein. The rationale behind the choice of these mutations is that T^{350} is necessary for 14-3-3 binding, as shown in the yeast two-hybrid assays (Figure 6C and D), so both HvABI5T^{350}A and C4Del will have a reduced capacity to interact with 14-3-3 proteins. Substitution of T^{350} with an aspartic acid (D) provides a negative charge at this position, thus mimicking the phosphorylated form of threonine. Since a phosphorylated interaction motif has a much higher affinity for 14-3-3 binding, we expect that trans-activation activity is retained by the

HvABI5T^{350}D protein. As shown in Figure 7B, the mutant proteins HvABI5T^{350}A and HvABI5C4Del have a reduced capacity (50%) to trans-activate the ABRC3-GUS. In contrast, the HvABI5T^{350}D mutant has the same activity as the wild-type form of HvABI5, providing support for our conclusion that an intact 14-3-3 binding motif at the C-terminus is important for the *in vivo* function of the HvABI5 protein.

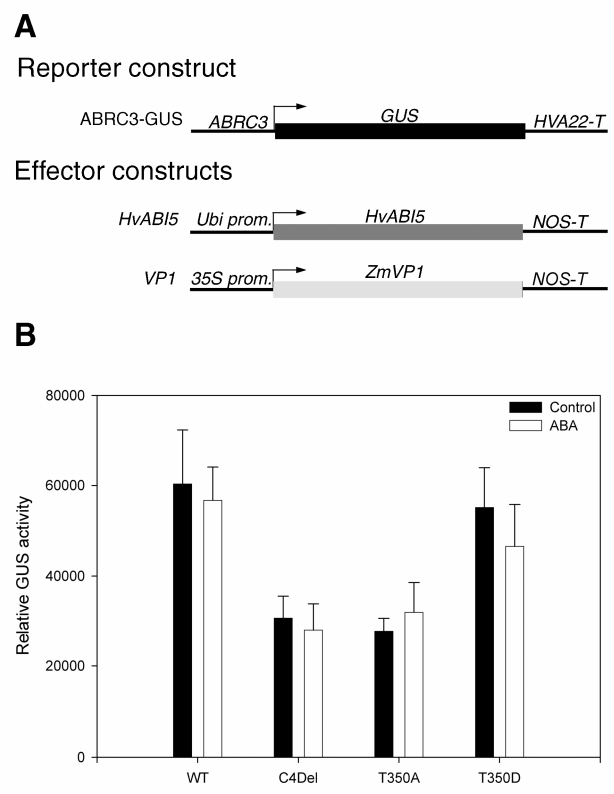

Figure 7. Trans-activation of the ABRC3-GUS by HvABI5 and VP1 is reduced when 14-3-3 binding motif is mutated in the HvABI5 protein.

(A) Schemes of reporter and effector constructs used in the transient expression assays. **(B)** To test the function of the C-terminal 14-3-3 binding motif, HvABI5 was substituted in a trans-activation experiment, for its mutant forms (C4Del, T^{350}A and T^{350}D). Untreated (solid bars) and ABA (20 µM) treated (white bars) barley seeds are shown (n=4) ±SE.

Finally, if the reduced trans-activation capacity of the HvABI5 proteins with non-functional 14-3-3 binding motif, is indeed the result of reduced 14-3-3 interaction, then we hypothesized that 14-3-3 silencing would also have a negative effect on the trans-activation activity of wild-type HvABI5 (in combination with ZmVP1). Figure 8 shows indeed that that co-expression of RNAi of all 14-3-3 isoforms negatively (up to 70%) affects trans-activation of the ABRC3-GUS by co-bombardment of HvABI5 and ZmVP1. Interestingly, the 14-3-3 isoforms A and B that do not interact with HvABI5, also show to be important in the VP1 and ABI5 induced trans-activation of the ABRC3-GUS (Figure 8). We hypothesize that is could be due to the fact that these isoforms play a role in binding VP1.

DISCUSSION

When a seed embryo has matured, it is important that the seed remains quiescent until conditions for germination become favorable. Seeds from some cereal crops have the tendency to germinate when still in the head (pre-harvest sprouting or *vivipary*), whereas others may show too much dormancy at harvest, which results in uneven germination. Both traits are undesirable from an agronomic viewpoint and may lead to serious economic losses. Abscisic acid biosynthesis and responses are clearly involved in both *vivipary* and dormancy (Koornneef et al., 2002). Although many elements of the ABA signal transduction cascade have been discovered in recent years (Rock, 2000; Schroeder et al., 2001; Finkelstein et al., 2002; Himmelbach et al., 2003), the picture is still far from complete. One new putative element, the 14-3-3 protein family, has come to the foreground in recent years as regulators of proteins that are important for ABA action during seed germination and stomatal regulation: the transcription factor VP1 (Schultz et al., 1998), plasma membrane K^+-channels (Van den Wijngaard et al., 2005), the vacuolar SV-channel (Van den Wijngaard et al., 2001; Peiter et al., 2005) and the plasma membrane H^+-ATPase (Kinoshita and Shimazaki, 2002; Brault et al., 2004). We used the barley primary root (radicle) and aleurone cells to get a better insight in the 14-3-3 / ABA relationship.

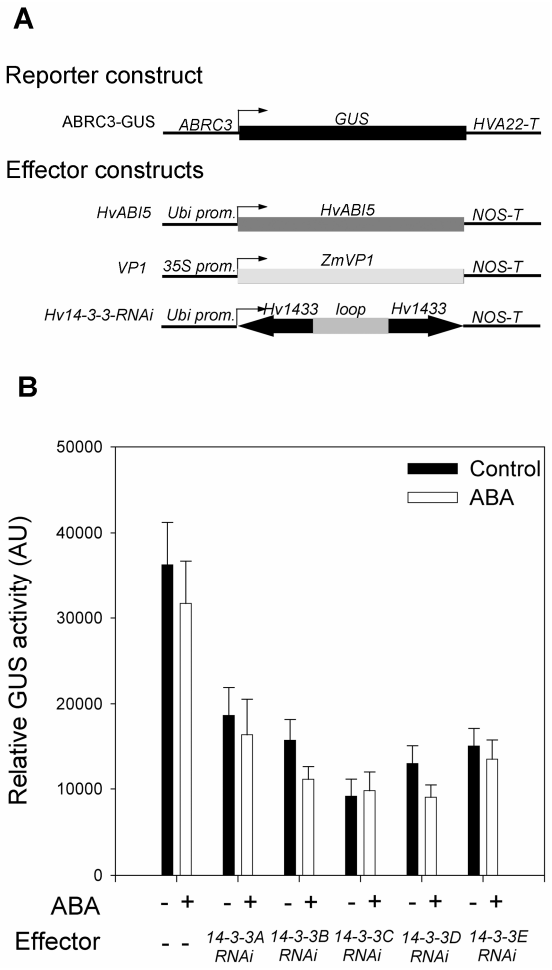

Figure 8. The HvABI5/VP1 mediated trans-activation of ABRC3-GUS is dependent on 14-3-3 proteins.

(A) Schemes of reporter and effector constructs used in the transient expression assays. **(B)** Embryo-less barley half seeds were bombarded with the ABRC3-GUS fusion together with the constitutively expressed transcription factors ZmVP1 and HvABI5. The role of 14-3-3 proteins in the VP1/ABI5 trans-activation was tested by co-bombarding isoform specific Hv14-3-3 RNAi constructs. Untreated (solid bars) and ABA (20 µM) treated (white bars) barley seeds are shown (n=4) ±SE.

Barley 14-3-3 genes and proteins respond to ABA

Our search for additional barley *14-3-3* genes resulted in the identification of two new isoforms, named *Hv14-3-3D* and *Hv14-3-3E*. The deduced amino acid sequence of the new 14-3-3 proteins showed highest homology between each other (75% identity) and Hv14-3-3D and Hv14-3-3E are orthologs of the wheat TaWIN2 and TaWIN1, respectively (Figure 1B). Barley radicle protrusion is an ideal system to study seed germination. Barley seeds are large seeds (approximately 8-9 mm) and thus radicles can easily be isolated from the germinating seed and can be studied as an isolated system. After a seed is imbibed in water, the plant maintains a post-germination developmental checkpoint for 2-3 days. When the seed experiences drought or salt stress within this period, it can respond with a growth arrest through the production of ABA (Lopez-Molina et al., 2001). When ABA is produced or applied outside this time window, there is no growth response. Therefore, we started the ABA treatments 22 hours after seed imbibition and measured growth and followed gene expression and protein levels of the 14-3-3 family. Expression of the *Hv14-3-3A* isoform is unaffected by ABA, but the protein shows an interesting ABA-dependent post-translational modification (Figure 3). Whereas in control radicles during the second day after imbibition, the 30 kDa Hv14-3-3A protein is fully truncated to a 28 kDa protein, ABA completely prevents proteolytic cleavage (Figure 3). The 28 kDa form of Hv14-3-3A lacks ten or twelve amino acids at the non-conserved C-terminus of the protein (Testerink et al., 2002). Although *Hv14-3-3B* and *Hv14-3-3C* are closely related genes (Figure 1B), their protein levels during development of the radicle differ greatly. Whereas the Hv14-3-3C protein appears only during a rather narrow time window, peaking at around 30h, the Hv14-3-3B protein remains stable throughout the second day (Figure 3). Moreover, ABA does not affect Hv14-3-3B protein levels, but now prevents the breakdown of the Hv14-3-3C protein that starts around 34 h (Figure 3). Isoforms D and E show similar patterns of expression and protein levels during normal development and in response to ABA treatment (Figures 2 and 3). ABA ensures that the D & E expression and protein levels are highly up-regulated in the course of the second day after imbibition. Clearly, there is a strong developmental and hormonal regulation of the barley 14-3-3s in an isoform-specific manner and it should be noted that the level of mRNA does not necessarily correlate with the corresponding protein level.

Function of barley 14-3-3 protein in ABA signaling

Since both 14-3-3 transcript and protein levels are strongly ABA-responsive, as described above, and in view of the well-documented function of 14-3-3 proteins in animal hormonal signaling (Thomas et al., 2005), we hypothesized that 14-3-3 proteins may be important for ABA signaling. To test this hypothesis we took advantage of the model system for studying the ABA signal transduction pathway in seeds: the barley aleurone layer (Lovegrove and Hooley, 2000). The cis-acting elements of the ABA-inducible *HVA1* gene (ABRC3) was chosen to report the effectiveness of ABA action and thus ABA signaling (Casaretto and Ho, 2003, 2005). From each 14-3-3 isoform, RNAi constructs were prepared (Figure 4A) and aleurone layers were co-bombarded with ABRC3-GUS constructs and 14-3-3 RNAi constructs. The outcome was rather surprising, silencing of each isoform resulted in a reduction of the ABRC3-GUS activity, which indicates that each individual 14-3-3 isoform has a key-position in the pathway leading from ABA perception to ABA induced gene expression.

A hallmark of 14-3-3 action is the interaction with other proteins. Our observation that silencing of the 14-3-3s negatively affects the progression of ABA signaling towards activation of gene expression, suggests that 14-3-3s interact with important mediators of ABA signaling. A yeast two-hybrid screen using five barley 14-3-3 isoforms as bait to screen a seven day old barley leaf cDNA library resulted in the identification of three members of the AREB/ABF/ABI5 family (HvABF1, HvABF2, and HvABF3). Since many bZIP transcription factor exist, >75 in *Arabidopsis*, and only four bZIP transcription factors are identified in the screen, it suggest that the 14-3-3 proteins specifically interact with the ABF family of bZIP transcription factors (Jakoby et al., 2002). Since the C-terminal end of most of the AREB/ABF/ABI5 like proteins contain the putative 14-3-3 interaction motif (Figure 2; supplementary material), we hypothesize that all of these proteins interact with the 14-3-3 protein family. To address the question whether the seed specific HvABI5 interacts with 14-3-3 proteins, and if so, whether there is isoform preference, we set-up a yeast two-hybrid assay (Figure 6C and D). Both the quantitative as well as the qualitative assay clearly shows that HvABI5 interacts with three of our 14-3-3 proteins: Hv14-3-3C, Hv14-3-3D and Hv14-3-3E. Despite the high degree of homology between the B and C isoforms (92% identity at amino acid level), B does not show an interaction with HvABI5 in our assay, which emphasizes the high degree of isoform specificity. A similar observation was made with the enzyme sucrose phosphate synthase (SPS) (Bornke, 2005) and from deletion analysis these authors concluded that differences in affinity for a certain target were mediated by the variable C-terminus of the 14-3-3s. Indeed, 50% of the last twenty

amino acids of 14-3-3B and 14-3-3C C-terminal end are different. We focused on the bZIP transcription factor HvABI5 as putative target for a number of reasons: i) HvABI5 has been shown to interact with the ABRC3 (Casaretto and Ho, 2003), ii) the HvABI5 protein sequence contains two canonical 14-3-3 interaction motifs (REIPT^{160}AP) and (RRTLT^{350}GP), where phosphorylation of T^{160} and/or T^{350} may control 14-3-3 interaction and iii) related bZIP-type transcription factors like AtABI5 and AtAREB1 are phosphorylated upon ABA treatment (Lopez-Molina et al., 2001; Furihata et al., 2006).

The serine or threonine residue in the canonical 14-3-3 binding motif (K/R-(x)-x-x-S/T-x-P) is important because (de)phosphorylation of these residues determines the affinity for 14-3-3 proteins. To investigate whether the two putative 14-3-3 binding motifs in the HvABI5 protein are indeed functional for 14-3-3 binding, we used site-directed mutagenesis to substitute the T^{160} and T^{350} for alanine residues. The site of interaction for the two strongest binding partners, Hv14-3-3D and E, is located at the C-terminal end around amino acid T^{350}. Intriguingly, substitution of T^{160} for an alanine only affects interaction with 14-3-3C and not with 14-3-3D & E. This suggests that 14-3-3C binds the HvABI5 protein in two different places. This two-site interaction resembles the way 14-3-3 proteins interact with AANAT (arylalkylamin N-acetyltransferase), the penultimate enzyme in melatonin biosynthesis (Ganguly et al., 2005). HvABI5 and AANAT also share the recently identified mode III binding motif (Coblitz et al., 2006) located at the very C-terminal end; RRTL<u>T</u>GPW-COOH and RRN<u>S</u>DR-COOH respectively. However, here we show that different 14-3-3 isoforms interact with one and the same target (HvABI5), but that they bind the target in a different fashion. It can be speculated that different mode of interaction between HvABI5 and the three 14-3-3s is functionally relevant, as HvABI5 is also involved in a positive regulatory loop up-regulating its own expression (Casaretto and Ho, 2005). These different binding modes, the fact that 14-3-3 proteins act both as homo- and heterodimers, and the possibility that the 14-3-3 proteins ensure coordinated regulation of the ABA signaling network through interaction with multiple intermediates, may be the reason that our silencing data suggest little redundancy in 14-3-3 action. In order to identify the complete 14-3-3 interactome from germinating barley seeds, we currently take a 14-3-3 affinity purification approach, using the recombinant 14-3-3 proteins and extracts from control and ABA treated seeds.

Intact C-terminal 14-3-3 interaction motif is important for trans-activation activity of HvABI5

Investigating the *in vivo* function of HvABI5 is possible through ectopic expression of the two transcription factors HvABI5 and ZmVP1, which results in a trans-activation of the ABRC3-GUS in the absence of ABA. Using this transient trans-activation method, we studied the relevance of the 14-3-3 interaction site (RRTLT^{350}GP) for *in vivo* activity. Mutant versions of HvABI5 (HvABI5T^{350}A & C4Del) that do not contain the 14-3-3 binding site, have a reduced ability to trans-activate the ABRC3-GUS (50%). This provides indirect evidence that 14-3-3s are important for the *in vivo* trans-activation activity of HvABI5, and this idea was corroborated by the negative effect of 14-3-3 silencing on the trans-activation activity of HvABI5 (Figure 8).

At first sight, the dependence of ABA signaling in seeds upon five individual 14-3-3 proteins seems bewildering. However, now that studies in the field of animal 14-3-3 biology have identified hundreds of 14-3-3 target proteins, a picture is emerging with a key position for 14-3-3 proteins in networks, like apoptosis, cell cycle control and hormonal signaling (Van Hemert, 2001; Thomas et al., 2005). We predict that the plant 14-3-3 proteome, just like the animal 14-3-3 proteome, will contain hundreds of target proteins as well (e.g. data from 14-3-3 affinity chromatography (Alexander and Morris, 2006) and unpublished data from our group). We hypothesize that a similar picture may come up for the ABA signaling network when the plant 14-3-3 proteome will near completion. There is certainly no lack of putative targets in the complex ABA signaling network, since many intermediates (Finkelstein et al., 2002; Rock, 2000) contain one or more canonical 14-3-3 interaction motifs. The challenge ahead will be to identify new 14-3-3 targets that have a function in the action of ABA and to understand what the involvement of multiple 14-3-3 proteins in the ABA signaling network means for the processes going on within the network and for the cross-talk with other signaling processes.

MATERIALS AND METHODS

Plant material

Two different varieties of barley (*Hordeum vulgare L.*) have been used for these studies. For expression profiling and Western-blots, variety Alexis was used (Saatzucht Jozef Breun GdbR, Herzogenaurach, Germany), and for biolistic transformation of aleurone layers the variety Himalaya was used (harvest 1998, Pullman Crop and Soil Sciences, Washington State University).

Isolation and growth of barley radicles

The isolation and growth of the barley radicles was performed as described in Van den Wijngaard et al., (2005).

Identification of Hv14-3-3D & Hv14-3-3E genes

To identify new barley 14-3-3 isoforms a degenerated primer (5'-RGAYTC5AC5YT5ATHATG-3', U.I.B. coding and where a 5 denotes an inosine) was designed against the homologues part of the 3' end of most of the known plant 14-3-3s. PCR was performed using this degenerated primer as forward and the 3' RACE primer as reverse. The amplified fragments were cloned into a pGEM-T (Promega) vector. Colonies were screened using filter lifting, and filters were hybridized with a radio labeled probe directed against the conserved region of the *Hv14-3-3C* cDNA. Positive clones were sequenced using the T7 promoter and Bigdye sequencing kit (Applied Biosystems). Performing 5' RACE the full-length cDNA sequence was identified. The full cDNA sequence was cloned in frame into the pPinpoint Xa (Promega) vector.

RNA isolation and Q-PCR

The isolation of total RNA from radicles and subsequent gene expression profiling by Q-PCR were described in Van den Wijngaard et al., (2005). Primers that were used for the Q-PCR can be found in Schoonheim et al., (2007).

Determination of 14-3-3 protein levels

The isolation of total protein and western-blotting were performed as described in Van den Wijngaard et al., (2005).

Preparation of DNA constructs

RNA interference constructs were designed to target the 3'UTR sequence of the five barley 14-3-3 isoforms. The five barley 14-3-3 sequences were aligned and primers were designed to the unique part of the 3'UTR. The primers of the 14-3-3A, B and C constructs were designed to introduce EcoRI restriction sites at the 5' end and BamHI restriction sites at the 3' end, for 14-3-3D and E vice versa. For 14-3-3A, B and C part of the coding region was used as loop DNA, for 14-3-3D and 14-3-3E part of the vector was used as loop DNA (pPinpoint-Xa). Amplicons were digested with EcoRI and ligated, the ligated PCR fragments (short-long) were inserted into a BamHI digested UBI1 driven vector (Ubi1-Luc from (Zentella et al., 2002)) resulting in the RNA interference construct. Primers that were used are published in Schoonheim et al., (2007). The reporter constructs UBI-Luc, ABRC3-

GUS, and UBI-GUS were described elsewhere (Casaretto and Ho, 2003). The 14-3-3E-GUS fusion was prepared by digesting the 14-3-3E-pinpoint construct and the UBI-GUS, with KpnI and SacI. The 14-3-3E insert was ligated at the 3' end of the construct between the GUS ORF and the Nos-T. The ABF-His constructs were prepared by digesting the Clontech pACT2 vector containing the ABF insert with BamHI and BglII. The digested insert was subsequently ligated into a BamHI digested Invitrogen pRSET-C vector. HvABI5 mutant constructs were prepared by PCR using primers that introduce the mutations. For the C-terminal four amino acid deletion mutant (C4Del) a TGA stop codon was inserted behind the L^{349}. To minimize PCR mistakes we digested the PCR fragment for the T^{350} mutants with SacII and KpnI, the digested PCR fragment was ligated into the SacII-KpnI digested UBI-HvABI5. For the HvABI5T^{160} mutants a two-step PCR was performed to introduce the SacII restriction site in the PCR fragment. Primer combinations that were used can be found in Schoonheim et al., (2007). To prepare the double mutant HvABI5, we digested the UBI-HvABI5T^{350}A and UBI-HvABI5T^{160}A with BamHI and SacII. The HvABI5T^{160}A BamHI-SacII fragment was ligated into the BamHI-SacII digested UBI-HvABI5T^{350}A vector. For yeast two-hybrid constructs the pGAL4-AD and the pGAL4-BD (Stratagene) were digested with the appropriate restriction enzymes. The inserts for most of the constructs were prepared by using PCR, for HvABI5 the constructs were prepared by digestion with SmaI and EcoRV and ligated into the SmaI site of the pBD-GAL4 vector. Primers that were used can be found in Schoonheim et al., (2007). All constructs were checked by sequencing.

Transient Expression Assays

Particle bombardment of embryo-less barley half seeds was performed according to Shen et al., (1993). Briefly, embryos of the barley cultivar Himalaya were cut off. The embryo-less barley seeds were imbibed in the dark at 20 °C for two days on top of moist filter paper. After two days the testa and pericarp layers were removed under a dissecting microscope. M17 tungsten particles (BIO-RAD) were coated with reporter and effector DNA (1:1) using 1 M $CaCl_2$ and 0.1 M Spermine (free base). Also an UBI-Luc construct was co-bombarded (1:1) with the reporter for the purpose of normalizing GUS activities. Twenty-four hours after bombardment the seeds are ground and enzymatic assays are performed in Shen et al., (1993). Every data point is the average of four bombardments ± SE. At least three individual experiments were performed.

Yeast two-hybrid

The Stratagene GAL4 Two-Hybrid phagemid vector kit was used. The improved yeast strain PJ69-4A was used (James et al., 1996), which contains an extra reporter selecting for adenine auxotrophy (*ADE2*). The *ADE2* is a more stringent reporter compared to the *HIS3*. Transformation of the yeast cells was performed with the lithium acetate method according to Gietz et al., (1992). Yeast two-hybrid screens were performed using a barley leaf yeast two-hybrid cDNA library that was a kind gift of Masumi Robertson (CSIRO Plant Industry, Canberra, Australia). DNA was isolated from interacting clones and inserts were sequenced using the pACT2 sequencing primer (Schoonheim et al., 2007). To double check interactions from the screen, DNA that was isolated from the interacting clones was re-transformed into PJ69-4A yeast. Double transformed yeast clones were grown up in 1 ml liquid medium (SD) lacking the amino acids Leucine and Tryptophan (-LW). Seven µl of the yeast culture was spotted on selective plates lacking the amino acids leucine, tryptophan, histidine and adenine (-LWHA) and grown for four days at 30 ºC (4 replicates). For the quantitative beta-galactosidase assays, 1 ml of yeast culture was spun down and used for activity assays (3 individual replicates). To check whether the clones contain the right DNA constructs, DNA was isolated from 3 ml of full-grown cultures. PCR was performed using vector and gene specific primers. The beta-galactosidase activity protocol was described at: http://www.fhcrc.org/labs/gottschling/yeast/Bgal.html. All Y2H data presented here were reproduced in at least three individual experiments.

Preparation of recombinant proteins and far-western analysis

The preparation of the recombinant proteins were described elsewhere (Van den Wijngaard et al., 2005). Far-western experiments were performed according to Moorhead et al., (1999), briefly; 1 µg of recombinant ABF-HIS protein was run on SDS-PAGE. Proteins were transferred to PVDF membranes using the BIO-RAD semi-dry blotting system (15V for 2 hours). Membranes were incubated with 5 % marvel for 16 hours at room temperature; subsequently membranes were incubated with 3 µg/ ml recombinant 14-3-3 for 2 hours. As secondary antibody we used Pierce avidin-HRP conjugate and HRP activity was visualized using the Amersham ECL detection kit.

Accession numbers

The sequences of the newly identified Hv14-3-3D, Hv14-3-3E, HvABF1, HvABF2, and HvABF3 were deposited in the NCBI database. Genbank accession numbers of the genes used in this study are as follows: HvABI5 (AY150676), *14-3-3A* (X62388), *14-3-3B*

(X93170), *14-3-3C* (Y14200), *14-3-3D* (DQ295785), *14-3-3E* (DQ295786), HvABF1 (DQ786408), HvABF2 (DQ786409), HvABF3 (DQ786410), *Actin* (AY145451), and *HVA1* (X78205).

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SUPPLEMENTARY MATERIAL

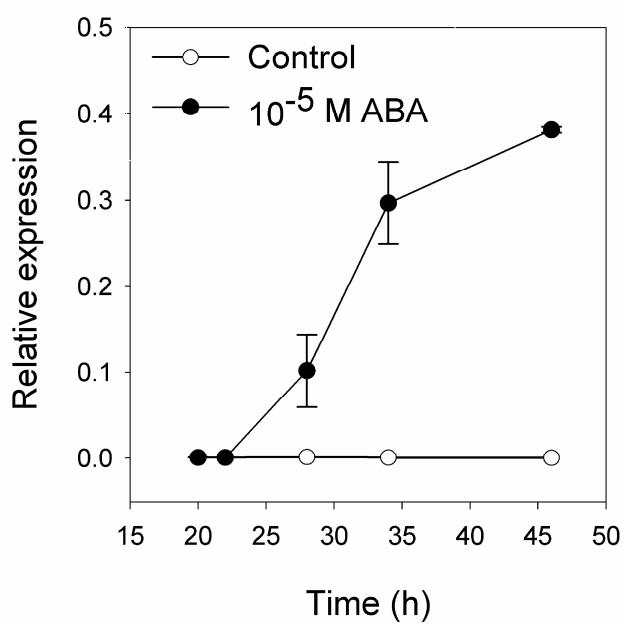

Figure 1. ABA strongly up-regulates the expression levels of the *HVA1* gene in elongating radicles.

Expression levels of the *HVA1* gene normalized on the housekeeping gene *actin* in growing radicles. The *HVA1* gene expression is followed in time (hours after imbibition) for untreated (○) and ABA (●; 10 µM) treated radicles.

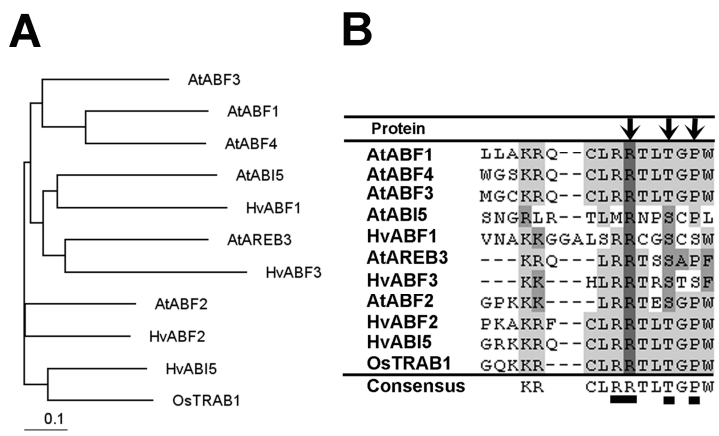

Figure 2. Sequence analysis of the ABF transcription factor family.

(A) Phylogenetic tree of ABF/AREB and ABI5 proteins from *Arabidopsis* and barley. **(B)** Closer look into the C-terminal tail of the proteins shows that the 14-3-3 motif (crucial residues underlined) is conserved.

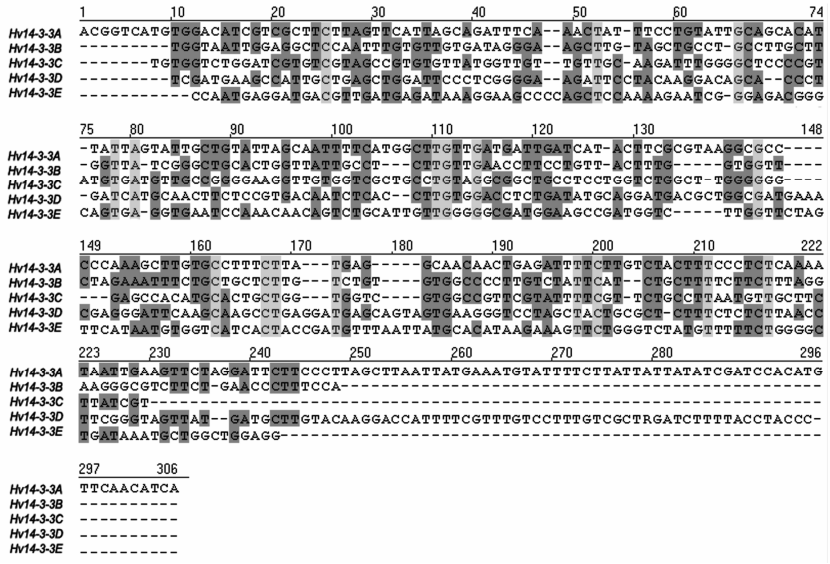

Figure 3. Sequence alignment of 3' UTR of 14-3-3 genes that was used for targeting of RNA interference

Chapter 3

Barley 14-3-3 proteins and GA signal transduction

With: Daniel da Costa Pereira, Holger Lill, and Albertus H. de Boer

ABSTRACT

Barley is the fourth ranked crop in the world (146 million tons), and is one of the most widely distributed cereal of the world. One of the important economical values of barley is that the grain is used in the malting process to produce beer and whisky. Uniformity and high percentage of seed germination are crucial factors of the malting process. Seed germination occurs when the environment of the seed is suitable for the development of the seedling. Factors like water availability, light, and nutrient composition of the soil are sensed and judged by the embryo. The balance of two antagonistic plant hormones, gibberellins (GA) and abscisic acid (ABA), determine whether the seed will germinate. In chapter 2, we showed that the highly conserved regulatory adapter proteins, 14-3-3 proteins, play an important role in the ABA signal transduction pathway. Evidence that 14-3-3 proteins play a role in the GA signal transduction pathway as well, is that 14-3-3 proteins have been shown to interact with the bZIP transcription factor REPRESSION OF SHOOT GROWTH (RSG). It was shown that 14-3-3 proteins 'inactivate' RSG by retaining the transcriptional activator of GA biosynthetic genes, in the cytosol. We hypothesized that if 14-3-3 protein levels would be reduced, GA biosynthesis would be activated due to the nuclear localization of RSG. In this study, we show that the barley orthologue of RSG (HvRF2A), interacts with all five barley 14-3-3 isoforms. However, silencing of the 14-3-3 isoforms did not result in promotion of the activity of the GA responsive alpha-amylase promoter in aleurone cells. In contrast, a reduction of the alpha-amylase promoter in the presence of exogenously applied GA_3 was observed, when transcript levels of each of the 14-3-3 isoform were reduced. These data show that 14-3-3 proteins, just like for the ABA pathway, play an important role in mediating the GA signal to downstream GA responsive target genes.

INTRODUCTION

Gibberellin history

Gibberelins (GAs) are diterpenoid compounds that were initially characterized by plant pathologists who studied the fungus infection of rice called bakanae (foolish seedling). The fungus that infected the rice seedlings caused a pale yellow, elongated seedling with slender leaves and stunted roots (www.plant-hormones.info). It was concluded that the fungus secretes a certain chemical that induces the foolish seedling phenotype. In 1938 Yabuta and Sumiki reported the first identification of the chemical that induces the foolish seedling phenotype and called it Gibberellin (Yabuta and Sumiki, 1938). Gibberellins were later found to be important phytohormones.

Gibberellins and seed germination

The plant hormone gibberellin regulates a variety of important plant processes like, stem elongation, leaf expansion, trichome development, flower development and seed germination (Olszewski et al., 2002). One of the best defined systems to study GA signal transduction is the cereal aleurone layer (Lovegrove and Hooley, 2000). At the start of seed germination, gibberellins are produced in the epithelium and developing shoot region (Kaneko et al., 2003). To promote seed germination, GAs are transported to the aleurone layer where they antagonize the effect of the dormancy hormone ABA and induce gene expression of a variety of hydrolytic enzymes (Kaneko et al., 2003). The hydrolytic enzymes are transported to the starchy endosperm where starch is hydrolysed into mobile sugars. The sugars are used as energy source by the embryo to develop from a heterotrophic organism to a chlorophyll containing autotrophic seedling.

Gibberellin perception

Until recently, GA perception in aleurone cells was thought to occur at the plasma membrane (Hooley et al., 1991). Membrane impermeable GA_4- Sepharose beads were shown to induce a high level of α-amylase (Hooley et al., 1991), and the suggestion of a plasma membrane localized receptor was corroborated by an experiment where micro-injected GA_4 was shown not to induce α-amylase promoter activity (Gilroy and Jones, 1994). Following GA perception, a calcium flux was observed that activates the GA signal transduction pathway (Gilroy and Jones, 1992). The Ca^{2+} fluxes are transient and are believed to occur as Ca^{2+} signatures (Allen et al., 2001). These Ca^{2+} signatures are decoded by many downstream Ca^{2+} binding proteins like, Calmodulin (CaM), calcineurin B-

like proteins (CBLs), and Ca^{2+} dependent protein kinases (CDPKs) (White and Broadley, 2003). In contrast to the indications that the GA receptor is localized at the plasma membrane as mentioned above, one of the most exciting revelations in this field was the identification of a soluble GA_4 receptor called GIBBERELLIN INSENSITIVE DWARF1 (GID1) (Ueguchi-Tanaka et al., 2005). The *GID1* gene encodes for an unknown protein that has similarity to the hormone-sensitive lipases (HSLs). The molecular function of GID1 is still to be elucidated, but the authors show that GID1 binds GA_4 in the micro-molar range and that the protein interacts with the DELLA protein SLR1 in a GA_4 dependent manner. SLR1 is epistatic to GID1, meaning that the loss-of-function *gid1-1/slr1-1* double mutant exhibits the SLENDER phenotype and not the GIBBERELLIN INSENSITIVE DWARF1 phenotype. This suggests that the GID1 and SLR1 function in the same pathway and that SLR1 is downstream of GID1 (Ueguchi-Tanaka et al., 2005). DELLA-domain proteins are transcriptional regulators that have been shown to repress the GA signal transduction pathway (Ikeda et al., 2001). The rice DELLA protein SLR1 is rapidly degraded upon GA_3 treatment in wild type plants and this GA_3 dependent degradation of SLR1 does not occur in *gid1-1* plants (Ueguchi-Tanaka et al., 2005). Knock-out mutants of the ortholog genes of *SLR1* in *Arabidopsis* (*GAI, RGA, Rht,* and *d8*) either do not show a phenotype at all or show only mild phenotypes. This suggests that the *Arabidopsis* DELLA proteins GAI, RGA, Rht, and d8 have a redundant function in the GA signal transduction pathway (Fleet and Sun, 2005).

GA induction of gene expression of hydrolytic enzymes in aleurone layer

Since the GID1 protein lacks a well defined functional homolog, the mechanism of how GID1 transduces the GA signal to the downstream effectors is still unknown. Because GID1 in yeast interacts with SLR1 in a GA_4 dependent manner, it is hypothesized that GID1 interaction with SLR1 promotes the ubiquitin / proteasome mediated breakdown of SLR1. The F-BOX protein OsGID2 / AtSLEEPY1, which is part of the E3 ligase complex SCF^{GID2}, binds to a phosphorylated form of SLR1 (Gomi et al., 2004). It is hypothesized that the SCF^{GID2} complex ubiquitinates the phosphorylated SLR1 protein and therefore initiates the proteasome mediated degradation (Sasaki et al., 2003). The GA_4/GID1 induced ubiquitin / proteasome mediated breakdown of SLR1 would result in the activation of the GA signal transduction pathway. Interestingly, in barley the GA induced degradation of HvSLN1 was shown to be phosphorylation dependent, since a tyrosine kinase inhibitor was capable of blocking the GA induced breakdown of SLN1 (Fu et al., 2002). More evidence for regulation on post-translational modifications was provided by Sasaki et al.

(2003), who showed that in *gid2-1* mutants the SLR1 protein is stabilized in a phosphorylated form (Sasaki et al., 2003). A third indication of an important role of post-translational modifications in the GA signal transduction pathway was the identification and characterization of the negative regulator of the GA pathway called SPY (Jacobsen and Olszewski, 1993; Robertson et al., 1998). *SPY* encodes a UDP-*N*-acetylglucosamine (*O*-GlcNAc transferase) and is hypothesized to attach *O*-GlcNAcylate to DELLA proteins and thereby modifying their activity / stability (Fleet and Sun, 2005).

14-3-3 proteins

14-3-3 proteins are acidic regulatory adapter proteins that form homo- and heterodimers. They play a multifunctional role in many different cellular processes, like carbon / nitrogen metabolism, cell division, apoptosis, and GA / ABA signal transduction (Van Hemert, 2001; Huber et al., 2002; Ishida et al., 2004; Schoonheim et al., 2007). The crystal structure of a 14-3-3 isoform has revealed that the 14-3-3 protein dimer consist of a clamp shape structure (Yaffe et al., 1997), which is capable of binding one protein in two different sites (Schoonheim et al., 2007) or binding two proteins at the same time (Braselmann and McCormick, 1995). The binding of the 14-3-3 proteins generally occurs at a conserved so-called 14-3-3 binding motif (R/K(x)xx$_p$T/$_p$SxP, where $_p$T and $_p$S denotes phosphorylated T or S). Two important proteins in the GA signal transduction pathway viz. SLR1 and GID2 do contain such so-called putative 14-3-3 binding motifs. In addition, as discussed above, the degradation of the HvSLN1 protein was shown to be phosphorylation dependent (Fu et al., 2002), which is another indication that 14-3-3 proteins could have a role in the GA signal transduction pathway. A third reason to study the role of 14-3-3 proteins in the GA pathway was that the tobacco 14-3-3 proteins have been shown to bind the bZIP transcription factor REPRESSION OF SHOOT GROWTH (RSG) (Ishida et al., 2004). RSG activates the expression of the ent-kaurene oxidase and therefore plays a role in the activation of the biosynthesis of biologically active GAs. 14-3-3 interaction with RSG results in cytosolic retention of the bZIP transcription factor where it is inactive (Ishida et al., 2004). The role of 14-3-3 proteins themselves in the GA biosynthetic pathway has never been studied. The evidence that 14-3-3 mediates the translocation of RSG, was shown by mutating a putative 14-3-3 binding motif in the RSG sequence, not by reduction of *in vivo* 14-3-3 protein levels. In this study, we focussed on the GA biosynthetic pathway from a different perspective namely ‘through the eyes’ of the 14-3-3 protein itself.

A good system to study the GA signal transduction pathway is the barley aleurone layer. The aleurone layer consists of one cell type and is highly responsive to GAs. Another

advantage of this cell layer is that they are easily transformed by particle bombardment, and do not produce GA biosynthetic enzymes themselves but do contain GA signal mediators like SLR1 (Kaneko et al., 2003). In this study we addressed two questions: I) Do the five barley 14-3-3 isoforms play an important role in the onset of GA biosynthesis since they control the cytosolic retention of the RSG transcription factor? II) Do the five barley 14-3-3 isoforms play a role in the activation or repression of the GA signal transduction pathway? We used transiently expressed RNA interference constructs to knock-down the expression levels of the five individual barley 14-3-3 genes and followed the activity of the co-expressed α-amylase promoter.

RESULTS

Hv14-3-3 gene expression and protein levels in GA_3 treated aleurone layers

Recently we have reported that the barley 14-3-3 protein family plays an important role in the ABA induced signal transduction pathway. The expression levels of the 14-3-3 isoforms respond to externally applied ABA in the germinating radicles and the different 14-3-3 proteins are recruited by ABA (Schoonheim et al., 2007). The two antagonizing hormones ABA and GA determine whether a seed will germinate or will stay dormant. To elucidate the role of the barley 14-3-3 protein family in the ABA antagonizing pathway, namely the GA pathway, we first studied the expression and protein levels in GA_3 treated aleurone layers. GA is produced in the barley embryo and subsequently transported to the aleurone layers where it induces a variety of hydrolytic enzymes to accommodate the process of seed germination. To minimize the endogenous GA level, embryo-less half seeds were used as model system. The embryo-less half seeds were imbibed in the dark for 2 days. Imbibed half seeds were treated with 10 μM GA, after 24 hours of treatment aleurone layers were removed from the endosperm and either total RNA was isolated or protein extract was prepared. Gene expression of the five barley isoforms *Hv14-3-3A, B, C, D, and E* were followed by Q-PCR (Figure 1). A 2.5 fold increase of transcript level was measured for *Hv14-3-3A*. Transcript levels of *Hv14-3-3B* was not affected by GA_3, however transcript levels of *Hv14-3-3C*, *D,* and *E* slightly decreased in response to GA_3 (Figure 1). Protein measurements on the aleurone layers were performed by western-blotting using 14-3-3 isoform specific antibodies (Figure 2). The protein levels of all five Hv14-3-3 isoforms do not respond to externally applied GA_3. The Hv14-3-3 isoforms A, B, and C are present in high amount whereas 14-3-3D and E are only present in small amounts (Figure 2).

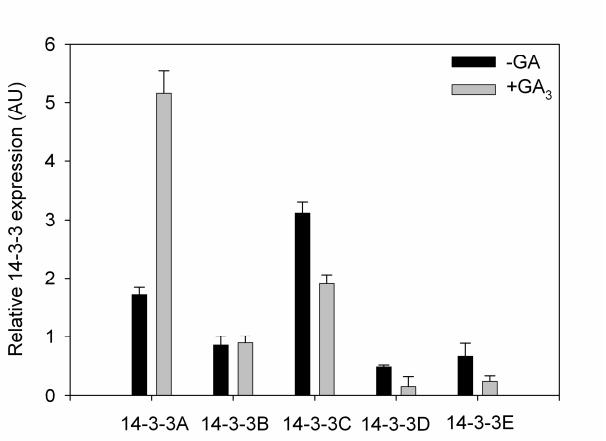

Figure 1. Gene expression profile of the barley 14-3-3 isoforms in untreated and GA_3 treated radicles.

Relative expression levels are measured using a Q-PCR, expression levels of untreated (solid bars) and GA_3 treated (grey bars; 10 µM) are normalized on the expression levels of the housekeeping gene Actin (n=3 ± SD).

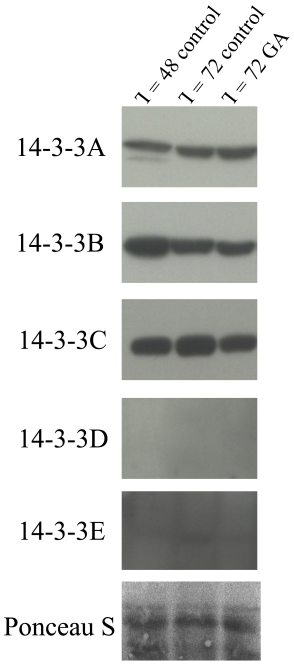

Figure 2. Protein levels of the five barley 14-3-3 isoforms in untreated and GA_3 treated barley aleurone layers.

Embryo-less half seeds were imbibed for 48 hours, subsequently treatments were given. After the time indicated, aleurone layers were isolated from the barley half seeds and protein extracts were prepared. Total protein extracts (30 µg) were run on SDS-PAGE and western-blotting was performed using isoform specific antibodies.

The role of 14-3-3 proteins in the GA signal transduction pathway

The interaction between the tobacco 14-3-3 protein family and the bZIP transcription factor REPRESSION OF SHOOT GROTH (RSG) has been studied extensively (Igarashi et al., 2001; Takahashi et al., 2003; Ishida et al., 2004). To confirm that also in barley the 14-3-3 proteins interact with this bZIP transcription factor we conducted a yeast two-hybrid assay using the 14-3-3 isoforms as bait and the barley RSG homolog as prey. Indeed as expected the barley homolog of the NtRSG protein viz. HvRF2a interacts with all five barley 14-3-3 isoforms (Figure 3).

To study the *in vivo* functional relevance of the barley 14-3-3 protein family in the GA biosynthetic and signal transduction pathway we used isoform specific RNA interference to

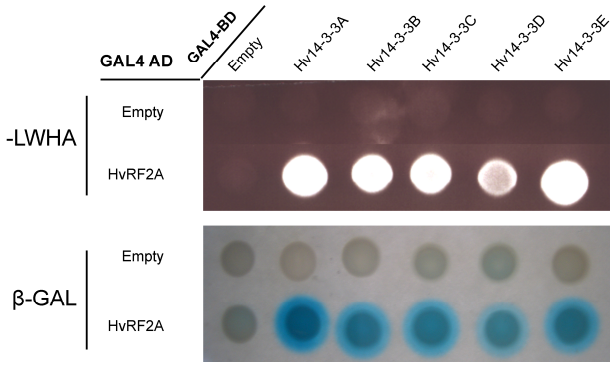

Figure 3. Interaction between the HvRF2A protein and the five barley 14-3-3 isoforms is shown using yeast two-hybrid assay.

In the upper panel (-LWHA) double transformants are grown on SD minus LWHA. In the lower panel (β-GAL) double transformants were grown on SD minus LW. The full grown spots were tested for β-Galactosidase activity. Results representative for at least three individual experiments are shown.

knock down the expression levels of each of the five barley 14-3-3 isoforms. One of the best characterized GA inducible promoters is the promoter of the α-amylase gene. A promoter-GUS fusion of the α-amylase32b (Amy32b-GUS) was used to follow the GA pathway in the aleurone cells of germinating barley seeds. Barley embryo-less half seeds were imbibed for two days to soften the two layers (testa and pericarp) that lie on top of the aleurone cells. Just before bombardment, the two layers were removed using tweezers and a dissecting microscope.

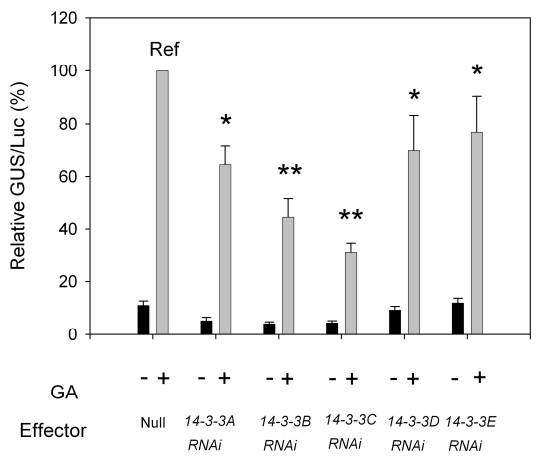

Figure 4. The GA_3 induced up-regulation of the α-amylase promoter is dependent on all five barley 14-3-3 isoforms.

The α-amylase promoter-GUS fusion was co-bombarded with 14-3-3 targeted RNAi constructs into barley aleurone cells. The barley half seeds were untreated (solid bars) and GA_3 treated (grey bars; 1 μM). To normalize the GUS activities, a constitutively expressed luciferase (UBI-Luc) construct is co-bombarded with the α-amylase promoter-GUS fusion. As control an empty UBI1 promoter construct was co-bombarded with the reporter constructs. For statistical analysis, the GUS/Luc ratios were ln-transformed. A one-way ANOVA showed a significant effect of silencing of each individual isoform compared to the empty construct (Null) ($P<0.001$). Silencing of the B and C isoforms differed significantly from silencing A, D, and E ($P<0.05$), but B and C were not significantly different from each other ($P>0.05$).

Subsequently, the aleurone cell layers were bombarded with the appropriate constructs. Exogenous GA_3 (1 μM) was applied to the barley half seeds, followed by an incubation for 24 hours. In our experiments the GA_3 treatments resulted in a 10 fold induction of the Amy32b-GUS compared to untreated barley half seeds (Figure 4). Since the 14-3-3 protein family has been shown to play an important role in the subcellular localization of the NtRSG protein (Igarashi et al., 2001), which is responsible for the biosynthesis of GAs (Fukazawa et al., 2000), we hypothesized that a knock down of 14-3-3 proteins would result in a nuclear localization of the RSG protein and thus an activation of the GA biosynthetic pathway. Using the amy32b-GUS construct as a reporter of the GA biosynthetic pathway, we could not detect any activation of GA biosynthesis due to the reduction of 14-3-3 gene expression (Figure 4, black bars). In the experiments where exogenous GA_3 was applied, we did find a significant effect of isoform specific 14-3-3 gene

silencing on the amy32b promoter activity (Figure 4, white bars). Moreover RNA interference of the two most homologs 14-3-3 protein isoforms B and C were found to have a significant larger effect on the Amy32b promoter than the isoforms A, D and E (Figure 4), indicating that the GA pathway contains mediators that have a 14-3-3 isoform specific preference.

14-3-3 protein targets in the GA_3 signal transduction pathway

The data presented above, indicates that the barley 14-3-3 protein family has an important function in mediating the GA signal to the alpha-amylase promoter. Because in our experiments the barley 14-3-3 proteins only showed a function in the GA signal transduction pathway downstream of the hormone itself, our second focus in this study was directed on the identification of novel 14-3-3 protein interactors in this part of the GA signal transduction chain. A potential 14-3-3 target is the GA suppressor HvSLN1. The HvSLN1 amino acid sequence contains a conserved putative 14-3-3 binding motif ('RRPIIS^{155}PP') and HvSLN1 is known to be phosphorylated. To test whether one or more of the five barley 14-3-3 isoforms interact with the HvSLN1 protein, we used the yeast two-hybrid principle. The HvSLN1 strongly auto-activates the reporter genes when cloned into the pBD-GAL4 vector, therefore it was swapped to the pAD-GAL4 vector in which it did not activate the reporter genes.

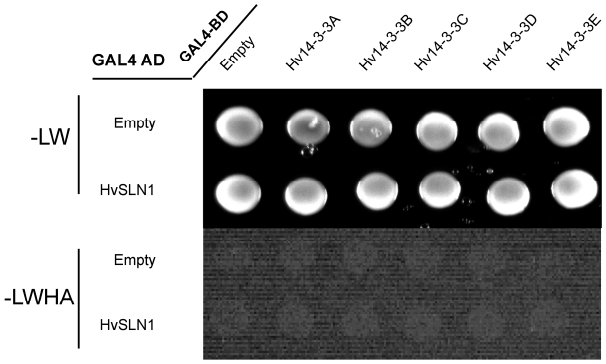

Figure 5. Yeast two-hybrid assay was performed to test whether HvSLN1 interacts with the barley 14-3-3 isoforms.

Yeast cultures are either grown on SD medium minus leucine and tryptophan to select for double transformants or grown on SD medium minus leucine, tryptophan, histidine, and adenine to test for the interaction between the bait and prey.

The yeast two-hybrid assay showed no interaction between the five barley 14-3-3 isoforms and the HvSLN1 protein (Figure 5). Simultaneously with the yeast two-hybrid assays, we conducted another experiment to test the *in vivo* function of the putative 14-3-3 motif of the HvSLN1 protein. 14-3-3 proteins generally bind target protein in the S/T amino acid inside the 14-3-3 binding motifs in a phosphorylation dependent manner. Therefore the serine in the putative 14-3-3 binding motif of HvSLN1 was mutated into an alanine (HvSLN1-S155A), that cannot be phosphorylated and thus 14-3-3 binding should not occur. Figure 6 shows that HvSLN1 protein is a strong inhibitor of the GA signal transduction pathway. The mutant form of HvSLN1 is also a strong inhibitor of the GA signal transduction pathway and results in comparable activity as its wild type form.

DISCUSSION

Plant embryos have the capability to wait for the right place and time to develop into an adult plant. The seed coat protects the embryo against extreme conditions (e.g. birds, UV, and drought). Sensing whether the growing embryo will survive in the existing environment is thought to be regulated by two antagonising signal transduction pathways of the two phyto-hormones ABA and GA (White and Rivin, 2000). Gibberellin biosynthesis is induced just prior to radicle emergence and activates a specific subset of genes to promote seed germination (Ogawa et al., 2003). As shown in *Arabidopsis* loss-of-function mutants, plants lacking GA biosynthetic genes are not capable of completing germination (Koornneef and Veen, 1980). Gibberellins are produced from geranylgeranyl diphosphate and as mentioned in the introduction, GAs consists of a large family of biologically inactive and active forms. The main active forms found *in planta* are GA_1 and GA_4 (Ogawa et al., 2003). The enzyme responsible for the conversion of biologically active GAs is the GA 3-oxidase. An important transcriptional regulator that regulates GA levels, and the expression of the GA3-oxidase, is the bZIP transcription factor RSG (Ishida et al., 2004). Loss-of-function mutants of *RSG* did not result in a phenotype although over-expression of the C-terminal bZIP domain resulted in a GA biosynthetic mutant dwarf phenotype (Fukazawa et al., 2000). The regulatory adapter protein 14-3-3 plays an important role in the translocation of the RSG protein in tobacco. The 14-3-3 interaction site, $RSLSVD^{116}$ in the RSG protein has been well characterized and was shown *in vivo* to be responsible for the GA and phosphorylation dependent cellular localization of the RSG protein (Ishida et al., 2004). Interestingly, the 14-3-3 binding site in RSG is located in the N-terminal stretch and this part of the protein is not well conserved in the rice and barley orthologs, the RF2A proteins.

However, the C-terminal half, containing the bZIP domain, is very well conserved. Although the putative 14-3-3 binding motif is not present in the barley RF2A protein, the protein does show an interaction with all five barley 14-3-3 isoforms in our yeast two-hybrid assay. This suggests that 14-3-3 proteins in barley play the same role for RF2A in barley as for RSG in tobacco.

An ideal system to study the GA biosynthetic and signal transduction pathway is the barley aleurone layer. The cells in the aleurone layer do not have GA biosynthetic activity and therefore are ideal to study how certain regulatory proteins are responsible for the activation of the GA biosynthetic pathway. Because 14-3-3 proteins have been shown to be a repressor of the GA biosynthetic pathway by retaining RSG into the cytosol, we designed an experiment where we reduced the expression levels of the 14-3-3 genes and followed the GA biosynthesis with the reporter gene α-amylase. In our experiments no activation of the GA pathway was observed (Figure 4). Since we do not have antibodies against the HvRF2A protein, the possibility exists that the HvRF2A protein is not present in the aleurone layer and therefore HvRF2A mediated activation of the GA biosynthetic route cannot occur. A second explanation for this observation is that HvRF2A is not the only regulatory protein that is necessary for the activation of the GA biosynthetic route. In contrast to the GA biosynthetic route, an interesting 14-3-3 dependency was found in the GA induced up-regulation of the α-amylase promoter when exogenous GA_3 was applied to the barley half seeds. The reduction of the α-amylase promoter by silencing the 14-3-3

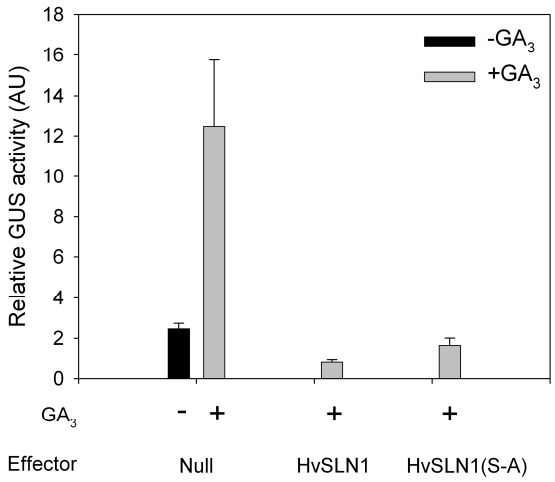

Figure 6. Over-expression of the HvSLN1 gene results in an inhibition of the GA signal transduction pathway.

Over-expression of the HvSLN1 wild type and HvSLN1-S^{155}A mutant was performed using the α-amylase promoter as reporter of the GA signal transduction pathway (n = 4 ± SEM).

genes has not been shown before. As discussed in the introduction, when GA is perceived by the HSL homolog GID1, an ubiquitin / proteasome mediated breakdown of the DELLA domain proteins is activated. The DELLA domain proteins are repressors of the GA signal transduction pathway and therefore GA triggers a repression of the repressors of its own pathway, thus activation. From the observation that 14-3-3 silencing in barley aleurone cells results in a reduction of the GA signal transduction, we concluded that some of these GA signal mediators are 14-3-3 interacting proteins. In cereals like rice and barley, only one gene encodes for a DELLA protein viz. SLR1 and SLN1, respectively. Because these two DELLA proteins contain a putative 14-3-3 binding motif RPIISP[156] that is conserved in both proteins, and both proteins are known to be phosphorylated, we considered them as potential 14-3-3 binding targets. Unfortunately, mutational analysis in combination with both over expression in the barley aleurone layer and yeast two-hybrid assays did not reveal any functional relevance of the putative 14-3-3 binding motif. Moreover, we could not detect any interaction between the barley 14-3-3 proteins and the SLN1 transcription factor. We conclude that 14-3-3 proteins play an important role in the mediation of the GA signal, and that more research has to identify 14-3-3 interacting proteins that have a function in GA signaling, to pinpoint the function of the 14-3-3 protein family in GA signal transduction pathway. Potential candidates are PKABA, GAMyb, HRT, and SPY1.

METHODS

Plant materials

Embryos of barley cv. Himalaya (Washington State University, Pullman, WA, harvest 1998) seeds were cut off and barley half seeds were surface sterilized by treatment with 10 % Glorix (commercial bleach without detergents). Subsequently half seeds were treated with 30 mM HCl for 2 minutes. Barley half seeds were imbibed for two days on top of a moist filter lying on top of vermiculite containing shooting buffer (20 mM NaSuccinate, 20 mM $CaCl_2$, pH = 7.0).

RNA isolation and Q-PCR

Total RNA was isolated from prepared aleurone layers using the hot borate protocol according to Wan and Wilkins, (1994). Briefly, approximately 15 aleurones were ground using mortar and pestle in liquid nitrogen. 500 µl of the 80 °C heated XT buffer (0,2 M NaBorate decahydrate (BORAX), 30 mM EGTA, 1 % SDS, 1 % NaDeoxychalate (Na-DOC), 35 µM Polyvinylpyrolidone (PVP), 10 mM DTT) was added to the powderized

aleurone tissue. Proteinase K treatment was performed and after several precipitation steps the quality of the total RNA was checked on a 1 % agarose gel. The synthesis of cDNA was performed using the Invitrogen superscript R.T. kit using an oligo dT primer. As template we used 2 µg of total RNA. Primers for the Q-PCR and the Q-PCR procedures have been described previously (Schoonheim et al., 2007), except that here Finnzymes SYBR green PCR mastermix was used.

Transient expression and reporter asays

Biolistic transformation was performed using a DuPont PDS/1000He system with rupture disks of 2200 PSI. The details of the particle bombardments together with the luciferase and GUS assays were described previously (Schoonheim et al., 2007).

Yeast two-hybrid assays

The Stratagene GAL4 two-hybrid phagemid vector kit was used. Yeast transformations were performed as described by Gietz et al., (1992). Details of the method have been described elsewhere (Schoonheim et al., 2007).

AKNOWLEDGEMENTS

We would like to thank Cor Zonneveld for the statistical analysis of the particle bombardment data. We also would like to thank Francesca Quattrocchio, for letting us use the DuPont PDS/1000He system for the particle bombardments.

Chapter 4

Characterization of the barley Two Pore Channel 1 (HvTPC1)

With: Paulien Gankema, Holger Lill, and Albertus H. de Boer

ABSTRACT

The *Arabidopsis* Two Pore Channel 1 (AtTPC1) has been recently identified as the gene that encodes for the well characterized calcium activated slow vacuolar (SV) channel. AtTPC1 was shown to play a role in the abscisic acid (ABA) signal transduction during seed germination. It was hypothesized that TPC1 functions as a calcium permeable channel that regulates the calcium influx during ABA signaling. In contrast, the rice and wheat TPC1 proteins were reported to be located at the plasma membrane. To elucidate the subcellular localization of the monocotyledonous TPC1 proteins, we focused on the barley TPC1 protein. The expression levels were studied in different barley developmental stages and in different tissues. The expression study showed that the *HvTPC1* transcript levels are relatively highly expressed in leaf tissue of barley seedlings. Localization studies using HvTPC1-yellow fluorescent protein (YFP) fusions did not result in a homogenous pattern. We hypothesize that the HvTPC1-YFP protein is mis-folded and therefore we observed cytosolic and nuclear localization of the membrane protein. To test whether HvTPC1 plays a role in ABA signaling during seed germination of barley we used the well characterized HVA1 promoter. The HVA1-promoter-GUS fusion was co-expressed with HvTPC1 generating RNAi. The reduction of *HvTPC1* by the means of RNA interference did not result in altered ABA promotion of the HVA1 promoter. In conclusion, *HvTPC1* shows a comparable expression pattern as the monocotyledonous *TPC1* genes. In our experiments, HvTPC1 does not regulate gene expression of ABA responsive genes.

INTRODUCTION

Calcium is an important second messenger in the cell. Many different abiotic and biotic stimuli like abscisic acid (ABA), gibberellin (GA), salinity, drought, hypo-osmotic stress, oxidative stress, and fungal elicitors (Sanders et al., 1999), evoke fast transient alterations in the free cytosolic calcium levels that result in the activation of a signal transduction pathway (McAinsh et al., 1992). The cytosolic calcium levels are strictly controlled by many different proteins, e.g. Ca^{2+} permeable channels, Ca^{2+}-ATPases, H^+/Ca^{2+} antiporters (CAX), calcium dependent protein kinases (CDPKs), calcineurin B –like proteins (CBLs), etc. (White and Broadley, 2003). The Ca^{2+} elevations occur in fast and transient peaks often referred to as transients (Trewavas and Malho, 1998). Transients are characterized by the frequency and amplitude of changes in Ca^{2+} concentrations. These Ca^{2+} transients are decoded by downstream target proteins that in turn activate other downstream signal mediators (Sanders et al., 2002). The main questions in this field are how these Ca^{2+} transients are established and how the specificity of the Ca^{2+} signature is encoded in these Ca^{2+} transients.

ABA induced calcium oscillations

As mentioned above, the hormone ABA induces Ca^{2+} transients. Mutants that have reduced ABA regulation (ABA insensitive), like the *abi1-1* and *abi2-1* mutants, show a reduced Ca^{2+} transient frequency in response to application of exogenous ABA (Allen et al., 1999). In contrast to these insensitive mutants, the hypersensitive mutant *era1-1* shows more frequent Ca^{2+} transients in response to exogenously applied ABA. Moreover it was shown that the hyperpolarization-activated calcium-permeable currents in the guard cell plasma membrane were elevated in *era1-1* mutants, as compared to wild type (Allen et al., 2002). These findings indicate that the ABA induced Ca^{2+} transients are regulated by the protein phosphatases ABI1 and ABI2, and the farnesyl transferase ERA1 and that these proteins act upstream of the Ca^{2+} transients.

Calcium oscillations and transport

Cytosolic Ca^{2+} transients can be established by either importing Ca^{2+} into or exporting Ca^{2+} out of the cytosol. A crucial part of the Ca^{2+} transients is the efflux of Ca^{2+} ions from the cytosol into the extracellular compartments, vacuole and / or other organelles. H^+/Ca^{2+} antiporters (CAX) and Ca^{2+}-ATPases are thought to play an important role in the Ca^{2+} efflux since Ca^{2+} is being transported against its chemical gradient, which costs energy

(Hirschi et al., 1996; White and Broadley, 2003). The import of Ca^{2+} into the cytosol can be performed in many different ways. As mentioned in the beginning of this introduction, many Ca^{2+} permeable channels are present in the membranes that are responsible for the Ca^{2+} influx. Since cytosolic Ca^{2+} concentrations $[Ca^{2+}]_{cyt}$ are much lower than the $[Ca^{2+}]$ in the apoplast and organelles, $[Ca^{2+}]_{cyt}$ can increase rapidly. The influx of Ca^{2+} ions into the cytosol is the first step of the Ca^{2+} transients and is tightly controlled by many different factors. Although the Ca^{2+} transients have been studied extensively for a long time, there are no reports, as far as we know of, that provide genetic evidence for a Ca^{2+} permeable channel that is responsible for the Ca^{2+} transients.

TPC1

Recently, it was shown that the two pore channel (TPC1) protein in *Arabidopsis* encodes a Ca^{2+} activated non-selective vacuolar channel (Peiter et al., 2005). Patch clamp experiments using protoplasts derived from *tpc1* loss-of-function mutants (*tpc1-2*) show that the TPC1 protein is responsible for the Slow Vacuolar (SV) current in the vacuolar membrane (Peiter et al., 2005). The authors show that *tpc1-2* plants have a reduced sensitivity towards exogenously applied ABA in the stage of seed germination. These data indicate that the TPC1 protein plays an important role in facilitating the Ca^{2+} influx from the vacuole that is necessary for mediation of the ABA signal. Interestingly, the *tpc1-2* mutant shows no abnormal ABA response in the regulation of stomatal closure, indicating that Ca^{2+} signaling and perception in stomata is different from that in germinating seeds (Peiter et al., 2005). An NCBI blast search using the rice TPC1 protein as bait results in four hits with E value of zero, viz. OsTPC1, HvTPC1, TaTPC1, and AtTPC1 (Figure 6).

Calcium transporters and 14-3-3 proteins

The SV channel in barley mesophyll vacuolar membranes was shown to be regulated by the regulatory adapter 14-3-3 protein (Van den Wijngaard et al., 2001). The activity of the SV current decreased upon incubation with recombinant Hv14-3-3B protein. Since Peiter et al. (2005) showed that the TPC1 protein is responsible for the SV current in *Arabidopsis* and because Van den Wijngaard et al. (2001) showed that the SV channel in barley is regulated by 14-3-3 proteins, we studied the interaction between the barley 14-3-3 proteins and the HvTPC1 protein. 14-3-3 proteins are acidic regulatory proteins that control the activity of target proteins. 14-3-3 proteins bind their target in a phosphorylation dependent manner and the binding generally occurs at a conserved binding motif called mode I: R/KSx_pS/$_p$TxP and mode II: R/Kxxx_pS/TxP (where $_p$S/$_p$T denotes a phosphorylated serine

or threonine) (Yaffe and Elia, 2001). The HvTPC1 protein consists of twelve trans-membrane helices and has the topology of two fused Shaker channels (Figure 3A and B). The HvTPC1 protein contains a conserved 14-3-3 binding motif I and is therefore a potential target for the 14-3-3 proteins. In this study we report that the HvTPC1 cytosolic loop between helix 6 and 7, interacts with the barley 14-3-3A isoform. Moreover we discuss the discrepancies that have been reported about the subcellular localization of the TPC1 proteins between monocot and eudicot species.

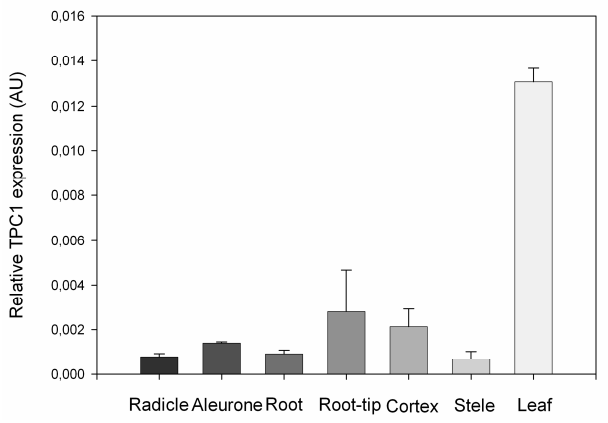

Figure 1. Expression profile of *HvTPC1* in different tissues of barley

Quantitative PCR was performed using cDNA prepared from RNA that was isolated from: barley radicles 22 hours after imbibition; Aleurone layers 48 hours after imbibition; roots, root-tip, cortex, stele, and leaf from 7 days old seedling. Relative expression levels are shown in arbitrary units, normalized on the expression of *Actin* (n=3 ± SD).

RESULTS

Expression profile of HvTPC1 in barley

Studies that have been performed on the cellular and subcellular localization of the TPC1 proteins from different monocot and eudicot species have raised the question whether the monocot TPC1 and the eudicot TPC1 proteins are orthologs. Reports about the rice *TPC1* gene have shown that the gene is mainly expressed in leaf tissue, shoots and cultured cells (Kurusu et al., 2004), whereas the *Arabidopsis TPC1* gene is expressed in all plant tissues and stages (Furuichi et al., 2001). For the *TPC1* gene in wheat it was reported that the expression levels are induced by treatment with the stress hormone ABA (Wang et al., 2005). In barley, the *TPC1* gene was cloned and published in the NCBI database in 2003, but no reports have been published on the characterization of the HvTPC1 protein. To investigate the gene expression of the *HvTPC1*, RNA was isolated from different barley tissues from different developmental stages. Samples were taken from 24 hours imbibed seeds of which the embryonic roots were isolated (radicle), 48 hours imbibed half seeds, of which the aleurone layer was isolated, 7 days old seedling of which the root, root-tip, cortex, stele, and leaf were isolated. The expression study of the *HvTPC1* gene revealed that the barley *TPC1* gene is mainly expressed in leaf tissue (Figure 1). This correlates well with the rice *TPC1* expression data that have been reported previously (Kurusu et al., 2004). This is in line with the fact that barley is closely related to rice. Although we observed the same expression pattern in barley as was reported in rice, we did not see an induction of the *HvTPC1* gene in ABA or salt treated radicles (data not shown) as was reported for the *TaTPC1* gene (Wang et al., 2005).

Localization of HvTPC1-YFP

Another puzzling observation concerning the monocot / eudicot TPC1 proteins is that subcellular localization studies have shown that AtTPC1 is located in the vacuolar membrane (Peiter et al., 2005) whereas the OsTPC1 was shown to be present in the plasma membrane (Hashimoto et al., 2005; Kurusu et al., 2005). The two proteins show 57% similarity at the amino acid level, and are therefore believed to be orthologs. Here, we used the barley aleurone to study the subcellular localization of the HvTPC1 protein. A yellow fluorescent protein (YFP) was fused to the carboxy terminus of the HvTPC1 protein and was introduced into barley aleurone cells by particle bombardment. As marker protein, we used the *Arabidopsis* Aleu protein fused to GFP. The Aleu protein is localized to the vacuoles (Fluckiger et al., 2003). As shown in Figure 2B, the Aleu-GFP fusion protein

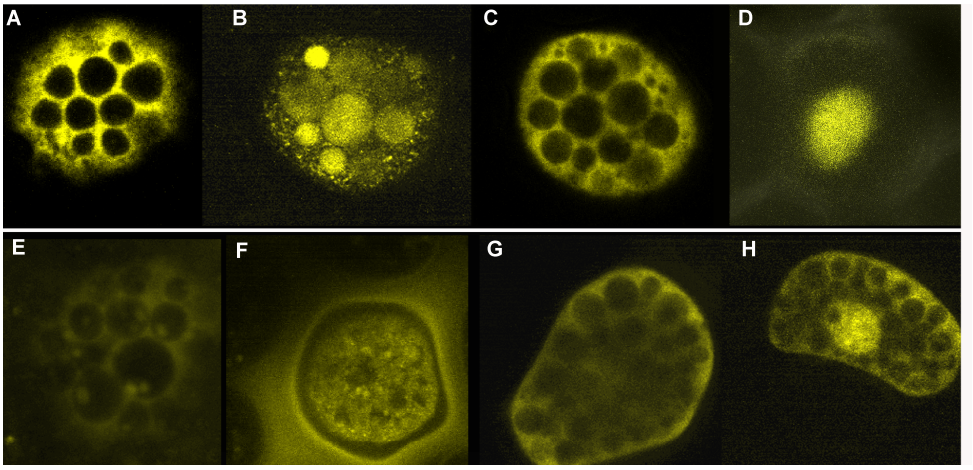

Figure 2. Subcellular localization of the HvTPC1-YFP fusion protein

Fusion constructs were introduced into barley aleurone cells using particle bombardment. **(A)** Free YFP shows a cytosolic localization. **(B)** Aleu-GFP is compartmentalized into the vacuoles and highlights the high amount of vacuoles present in the aleurone cell. **(C)** Hv14-3-3E-YFP fusion protein localized to the cytosol. **(D)** HvABI5-YFP fusion protein localizes to the nucleus. **(E-H)** HvTPC1-YFP does not show a homogenous picture of localization. In most cases the fusion protein localized to the cytosol, but in some cases localized to the nucleus.

marks the small vacuoles typically present in the barley aleurone cells. To our surprise, the HvTPC1-YFP fusion protein did not localize to any specific membrane and occasionally was nuclear localized (Figure 2E-H). The presence of HvTPC1-YFP fusion proteins was much lower than that of the other YFP proteins used in this study. We hypothesize that the HvTPC1-YFP fusion protein is either not folded correctly or is degraded rapidly in the aleurone cell and therefore shows no membrane localization.

14-3-3 interacts with cytosolic loop of HvTPC1

The HvTPC1 protein consists of twelve trans-membrane helices and has two pores in between helix 5-6 and helix 11-12 (Figure 3A and B). Between helix 6 and 7, a large cytosolic domain (125 amino acids) is present that contains two putative EF hands. Sandwiched between these two EF hands, a conserved putative 14-3-3 binding motif is found around the S^{372}: KYR_pSLP^{374} (where the $_pS$ denotes a phosphorylated Serine). Interestingly, the *Arabidopsis* TPC1 protein lacks the lysine, located in the 14-3-3 motif. The putative 14-3-3 interaction motif is conserved in all monocot TPC1 proteins (Figure 3C). To study this cytosolic domain, PCR was performed and the DNA that encodes for the

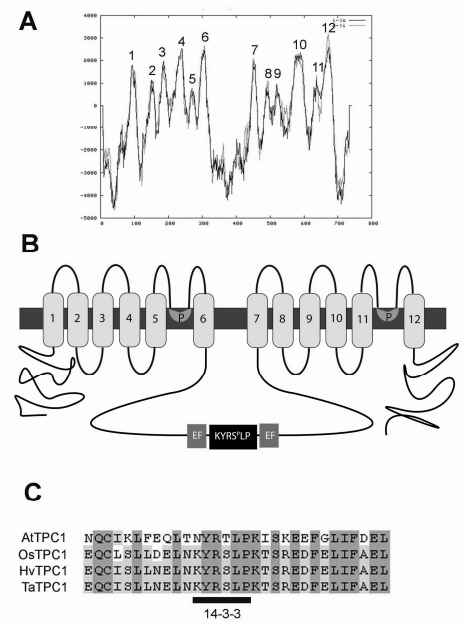

Figure 3. Topology of the HvTPC1 protein shows that the protein consists of two pores and twelve trans-membrane domains.

(A) The protein sequence was analyzed using the TMpred (ch.embnet.org) transmembrane prediction server. Transmembrane helices are numbered 1-12. **(B)** The transmembrane prediction of the TMpred extrapolated into a scheme. **(C)** Focussed on the putative 14-3-3 binding motif located in the cytosolic domain between transmembrane helix 6 and 7.

amino acids 315 to 436 were amplified and cloned into the GAL4-BD vector. To study the potential interaction with the five barley 14-3-3 isoforms, a yeast two hybrid assay was performed. Interestingly, one of the five barley isoforms shows interaction with the cytosolic domain of the HvTPC1 protein, namely Hv14-3-3A (Figure 4). The interaction between the cytosolic loop of HvTPC1 activates the *His3* reporter gene but does not activate the *Ade2* and *LacZ* reporter genes. These data indicate that Hv14-3-3A interacts only weakly with the cytosolic loop of HvTPC1.

The role of HvTPC1 in the ABA signal transduction pathway

As mentioned in the introduction, the AtTPC1 protein has been shown to function as mediator of the ABA signal transduction pathway. The data of Peiter et al. (2005) suggest that the TPC1 protein plays a role in the release of calcium into the cytosol, which

subsequently activates the ABA signal transduction pathway. To test whether the barley TPC1 protein also plays a role in the ABA signal transduction pathway, we used RNA interference to knock-down the expression of the *HvTPC1* gene in barley aleurone cells. As reporter of the ABA signal transduction pathway, the HVA1 promoter was fused to GUS and the constructs were introduced into the barley aleurone cells by particle bombardment. As shown in Figure 5, the reduction of *HvTPC1* expression in the aleurone by the means of RNA interference did not result in an alteration of the characteristics of the HVA1 promoter in the absence or presence of ABA. This suggests that HvTPC1 in barley aleurone cells does not facilitate the Ca^{2+} transients that are necessary for the activation of the ABA signal transduction pathway.

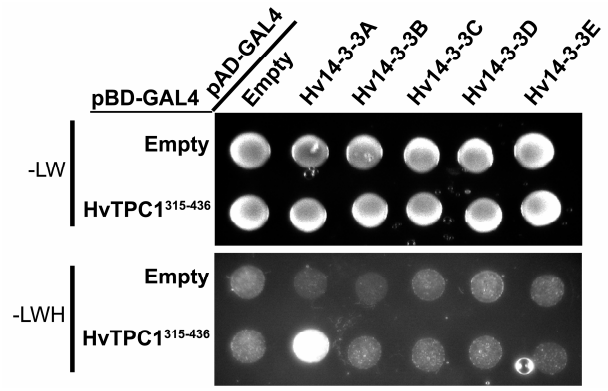

Figure 4. Yeast two-hybrid assay between cytosolic domain and barley 14-3-3 isoforms
The cytosolic domain of the HvTPC1 protein was fused to the GAL4- binding domain, and was co-expressed with 14-3-3-AD fusions. The double transformed yeast cells were tested for the activation of the *HIS3* reporter gene by growing them on SD plates lacking the His amino acid (SD minus LWH).

DISCUSSION

It is hypothesized that during evolution Ca^{2+} has evolved into such an important second messenger, due to the necessity to maintain low $[Ca^{2+}]_{cyt}$. Cells have to maintain low levels $[Ca^{2+}]_{cyt}$ due to the fundamental chemical properties of the solubility of Ca^{2+} in the presence of orthophosphate (P_i) (Sanders et al., 1999). Because resting $[Ca^{2+}]_{cyt}$ are low (100-300 nM), it is rather easy to rapidly increase the $[Ca^{2+}]_{cyt}$ by ten to twenty-fold (Sanders et al., 1999). Since the $[Ca^{2+}]_{cyt}$ regulates a variety of different signal transduction pathway, like development, ABA, GA, fungal elicitors, nodulation (Nod) factors

(Sanders et al., 2002), it is important that $[Ca^{2+}]_{cyt}$ is strictly regulated.

In the last decades many Ca^{2+} permeable channels and pumps located at the tonoplast, plasma membrane, endoplasmic reticulum, chloroplast and nuclear membranes have been identified using electrophysiology (White, 2000). Recently, one of these Ca^{2+} permeable channels that was already characterized based on its current, using electrophysiology, is now also characterized on a genetic level. The SV current was shown to be established by the Ca^{2+} -dependent Ca^{2+} -release channel called Two Pore Channel 1 (*TPC1*) (Peiter et al., 2005). Loss-of-function and gain-of-function mutants showed that the TPC1 protein is responsible for the SV current measured in the tonoplast of *Arabidopsis*.

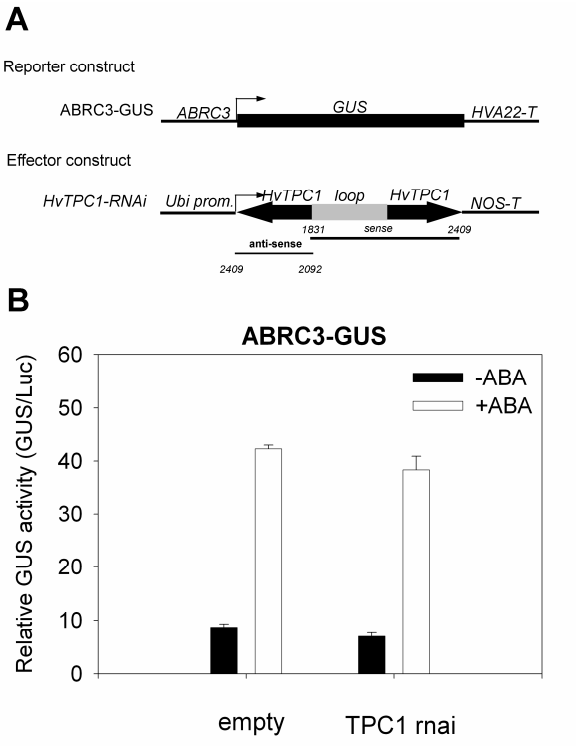

Figure 5. Gene silencing of *HvTPC1* does not alter the ABA activation of the ABRC3-GUS
Biolistic transformation of barley aleurone cells was performed. The ABRC3-GUS fusion together with the UBI-Luc as internal standard functions as reporter of the ABA induction of gene expression. Co-expression of the *HvTPC1* targeted RNAi construct did not result in alteration of the activity of the ABRC3-GUS in the absence (solid bars) or presence of ABA (open bars) (n=4 ± SEM).

The NCBI database contains four genes encoding TPC1 proteins, which belong to *Arabidopsis*, wheat, rice, and barley. The identity between the barley TPC1 protein and the other three proteins are 57%, 97%, and 84%, respectively (Figure 6). Intriguingly, in contrast to the *Arabidopsis* TPC1 protein, the rice TPC1 has been shown to be localized in the plasma membrane. In this study, we focused on the barley TPC1 protein to investigate its function and to study its subcellular localization. First, we studied the gene expression profile of the *HvTPC1* gene in different kinds of tissue of barley. This profile showed that the *HvTPC1* expression is relatively high in leaves as compared to other tissues (Figure 1). These data correlate with that of the rice *TPC1*, as is to be expected if we look at the sequence conservation between the monocotyledonous plants (Figure 6). Nevertheless, the expression level of the *HvTPC1* in radicles was not enhanced by treatment with ABA (data not shown), as reported for the wheat *TPC1* gene in ABA treated roots (Wang et al., 2005).

An important question to be resolved for the TPC1 channel remains the localization of the TPC1 protein. Therefore, HvTPC1-YFP fusions were prepared and were over-expressed in barley aleurone cells. Unfortunately, when using membrane localized proteins like the KCO1-GFP (tonoplast marker) fusion protein and the TPC1-YFP, no membrane localized signal could be detected. It seems that the barley aleurone layer has some problems synthesizing the membrane localized proteins fused to either GFP or YFP. Interestingly, when the vacuolar marker Aleu-GFP was used, many vacuoles could be visualized (Figure 2), showing that vacuoles are present, and that the transient expression system works.

The AtTPC1 protein was shown to encode for the channel that is characteristic for the SV current in vacuolar membranes and in barley it was shown to be regulated by the regulatory adapter proteins 14-3-3 (Van den Wijngaard et al., 2001; Sinnige et al., 2005b). Therefore, we tested whether the cytosolic domain of the HvTPC1 protein, which contains a putative conserved 14-3-3 binding motif, also interacts with 14-3-3 proteins. The domain between helix 6 and 7 (Figure 3) was tested in a yeast two-hybrid assay. To our surprise, only the Hv14-3-3A isoform showed interaction with the cytosolic domain (Figure 4). This is a puzzling observation since the SV current in barley protoplasts was shown to be regulated by either 14-3-3B or 14-3-3C but not by 14-3-3A (Sinnige et al., 2005b). These data suggest that either the 14-3-3 isoforms B and C bind the HvTPC1 protein at the N or C terminus, or it suggests that the HvTPC1 protein does not encode for the SV channel in barley. To study this in further detail, barley protoplasts lacking the HvTPC1 protein should be examined for the presence of the SV current.

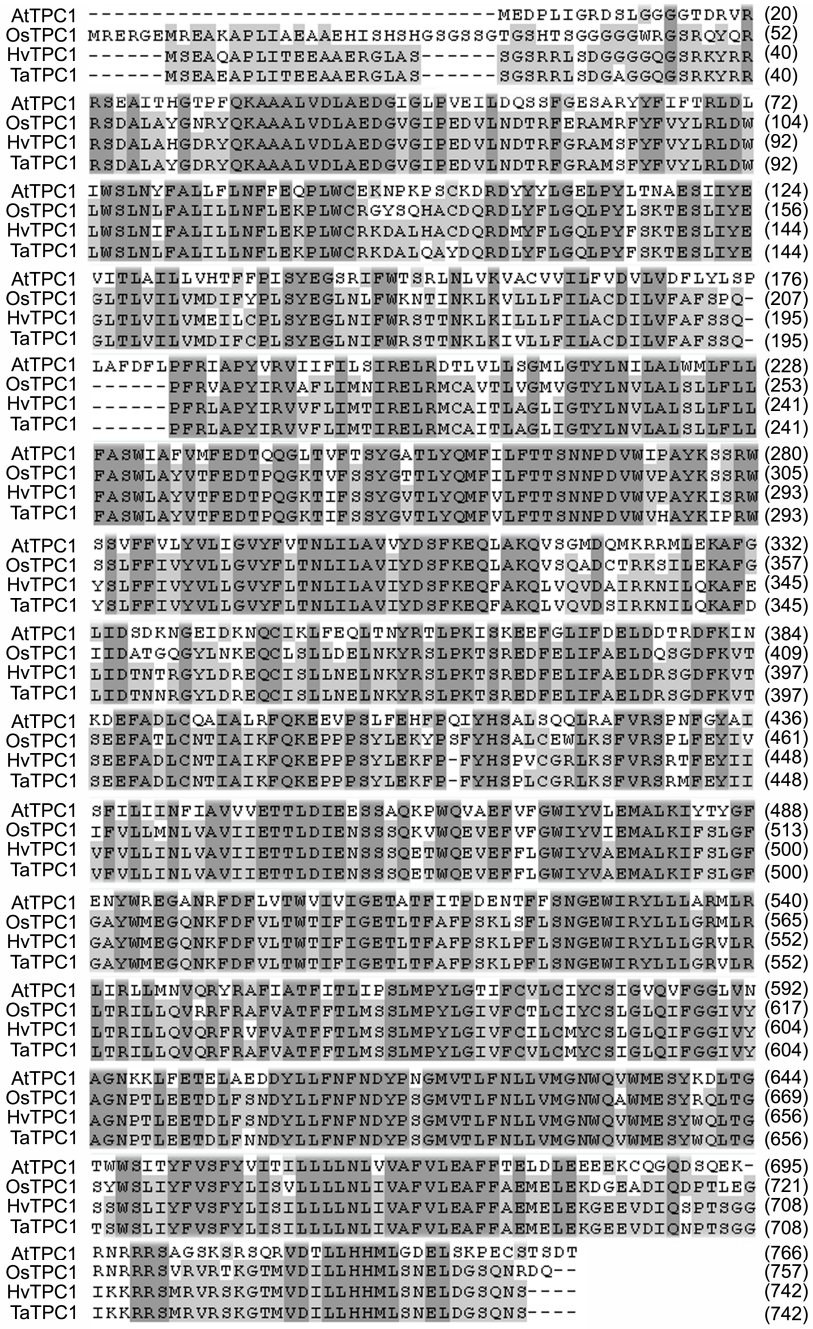

Figure 6. Amino acid alignment of *TPC1* genes of *Arabidopsis, rice, barley, and wheat.*

Residues highlighted in dark grey background are conserved in all 4 species, highlighted in light grey are conserved in two or more species and residues in white background are not conserved.

Another characteristic of the *Arabidopsis* TPC1 protein is that it has been shown to be important in the ABA signal transduction pathway. The *tpc1-2* loss-of-function mutants show a reduced sensitivity to exogenously applied ABA. Interestingly, this reduced ABA response is only noticeable during seed germination but not at the level of stomata regulation (Peiter et al., 2005). To test whether the barley TPC1 protein is also a mediator of the ABA response we tested its functionality in the activation of the promoter of the ABA inducible gene HVA1 (ABRC3). The ABRC3-GUS fusion was used to measure promoter activity in the absence and presence of ABA. *TPC1* transcripts were reduced by co-expressing RNA interference constructs targeted against the *HvTPC1* gene. The RNAi experiments did not result in an alteration of the ABRC3-GUS activity. This suggests that in aleurone cells, HvTPC1 does not mediate the ABA signal towards the ABRC3. Further research has to be performed using *TPC1* loss-of-function barley plants to unravel the localization and functions of the barley TPC1 protein.

METHODS

RNA isolation and Q-PCR

Total RNA was isolated using Invitrogen Trizol reagent. Plant tissue was homogenized with mortar and pestle using liquid nitrogen. After Trizol extraction, two times phenol-chloroform purification was performed and subsequently total RNA was precipitated using 2-propanol. The pellet was dissolved in TE buffer and DNAse treatment was performed. Another series of phenol-chloroform purification was performed and RNA was again precipitated with 2-propanol. The RNA samples were quantified by measuring the absorbance at 280 nm. The quality of RNA was checked by running one µg of total RNA on an ethidium bromide gel and checking for intact ribosomal RNA bands. cDNA was synthesized from 1-5 µg total RNA using the invitrogen superscript RT ®. Quantitative PCR was performed using the MJ-Research Opticon monitor DNA engine and using the Finnzymes SYBR green PCR mastermix. Q-PCR experiments were done with the following primer combinations: HvActin FW (5'- GTATGGAAACATCGTGCTCAGTGG-3') vs. HvActin RV (5'-CTTGATCTTCATGCTGCTCGGA-3'), and HvTPC1 FW (5'-TTGCTGAGATGGAACTGGAGAAAGG-3') vs. HvTPC1 RV (5'-ACCATCGTACCCTTTGACCTCACAC-3').

Preparation of UBI-HvTPC1-YFP

An EST clone containing three quarters of the *HvTPC1* gene was a kind gift of Dr. Thordal-Christensen (RVAU, Copenhagen, Denmark). The three prime end of the *HvTPC1* gene was cloned by PCR using leaf cDNA as template and using the following primer combination HvTPC1 (a.a. 315) FW (5'- GCTGTCATCTATGATAGCTTTAAGG-3') vs. HvTPC1 RV (5'- CAAGAGTTTTGAGATCCATCAAGTTC-3'). This PCR fragment was cloned into the Promega pGEMT-easy vector. This vector was digested with HindIII and SacII, and the resulting fragment was ligated into the EST clone that contained the five prime end of the gene. The full length HvTPC1 was cut out of its vector with AscI and SacII, this fragment was diluted fifty times and was used as template for another PCR. PCR was performed using HvTPC1 FW BamHI (5'-TCAGGATCCATGAGCGAAGCGCAGGC-3') vs. HvTPC1 RV SacII (5'-TTACCGCGGACAAGAGTTTTGAGATCCATCAAGTTC-3'). Parallel to this PCR also a reaction was performed using the YFP gene as template and using the YFP FW SacII (5'-GGAGCCGCGGATGGTGAGCAAGGGCGAG-3') vs. YFP RV BamH1 (5'-CTTTGTTAGCAGCCGGATCCC-3'). Both reactions were digested with the appropriate restriction enzymes. Ligation was performed of the two fragments resulting in an HvTPC1 carboxy terminus fusion with the YFP gene. This fusion was again digested with BamHI and subsequently ligated into a UBI1 promoter driven vector.

Transient expression of HvTPC1-YFP by particle bombardment

The UBI-HvTPC1-YFP construct was introduced into barley aleurone cells by particle bombardment using a DUPONT PDS1000. The detailed protocol has been described before in Schoonheim et al., (2007). Confocal microscopy was performed using the Nicon eclipse TE2000-S microscope in combination with the BIO-RAD Radiance 2000 laser scanning system.

ACKNOWLEDGEMENTS

We would like to thank Dr. Hans Thordal-Christensen for the kind gift of the overlapping *HvTPC1* EST clones, which were partially used to clone the full length *HvTPC1*. We would also like to thank Marijke Wagner for help with the confocal laser scanning microscope.

Chapter 5

14-3-3 affinity purification in barley embryos

With: Daniel da Costa Pereira, Giulia Friso, Klaas Jan van Wijk, and Albertus de Boer

ABSTRACT

In recent studies, we have shown that the regulatory adapter proteins, 14-3-3 proteins, play an important role in the regulation of seed germination. The highly conserved 14-3-3 proteins were shown to be necessary for the promotion of gene expression by the plant hormones abscisic acid (ABA) and gibberellin (GA). Since barley seeds are large (0.8 – 1.0 cm) it is feasible to dissect seed embryos and isolate embryonic proteins. To identify novel 14-3-3 targets in the hormonal signal transduction pathways important for the regulation of seed germination, we conducted large scale 14-3-3 affinity purification using embryonic protein extract. The R18 peptide, which binds all 14-3-3 isoforms with high affinity, was used to specifically elute target proteins from the 14-3-3 affinity column. Proteins in the R18 and mock elution were run on SDS-PAGE and were identified using nano LC-ESI-MS/MS. 14-3-3 interactions were confirmed by performing far-western analysis, the data presented show that the targets directly interact with 14-3-3C proteins. Twelve proteins were identified as 14-3-3 interactors, of which ten proteins were previously identified in a similar 14-3-3 affinity screen using proteins extract derived from barley leaves. Four of the ten previously identified 14-3-3 interactors were shown to be different types of invertases. The two novel 14-3-3 interacting proteins were identified, namely: RuBisCO activase and nucleolysin TIAR.

INTRODUCTION

Seed germination

Seed germination and subsequent development of the heterotrophic embryo into an autotrophic seedling is a critical phase of plant development. This process is strictly regulated by water availability and other environmental cues like light, nutrient composition of the soil, quality of the water, and day length (Bewley, 1997; Koornneef et al., 2002). The process of seed germination can be divided into three phases. I) the embryo takes up water and subsequently metabolic activity is recovered, II) cell division in the primary root (radicle) prepares the radicle for protrusion through the coleorhizae, III) cells in the radicle elongate, resulting in the protrusion of the radicle through the coleorhiza (visible germination). This marks the point of no return and the embryo will develop into an autonomous photosynthetic seedling (Bewley, 1997; Lopez-Molina et al., 2001).

When the embryo enters the first phase of germination, the levels of the two antagonistic hormones, gibberellin (GA) and abscisic acid (ABA), determine whether the embryo will pursue the process of germination (Garciarrubio et al., 1997; Finkelstein and Lynch, 2000a). When the environment is suitable, ABA levels drop and gibberellin levels rise (Iglesias and Babiano, 1997). Gibberellins are subsequently exported out of the embryo to the aleurone cells where it induces gene expression and subsequent secretion of a variety of hydrolytic enzymes, e.g. α-amylase. In contrast, when the environment is not suitable for seed germination (e.g. salinity stress), the embryo will produce ABA and is prevented from entering the second phase of seed germination. The dormant embryo will induce a different subset of genes, e.g. *HVA1*, *HVA22*, and *Em* to adapt to the environment (Shen, 1996; Carles et al., 2002). Seed germination will occur when the cell is fully adapted to the environment and subsequently hormone levels will change accordingly.

In previous studies performed in our laboratory, it was shown that the regulatory adapter protein family, called 14-3-3 proteins, plays an important role in the regulation of promoter activities of genes under the control of ABA (Schoonheim et al., 2007) and GA (chapter 3).

14-3-3 proteins

14-3-3 proteins are regulatory adapter proteins that are conserved throughout all eukaryotic organisms (Aitken, 2006). An interesting feature of 14-3-3 protein family is that they regulate a variety of molecular targets in one cell. The interaction between 14-3-3 proteins and its interactors is in most cases switched 'on' or 'off' by phosphorylation of a target sequence (Fu et al., 2000; Van Hemert, 2001; Aitken, 2006).

In view of the afore mentioned function of 14-3-3s in ABA and GA signaling, we addressed in this study the question which 14-3-3 interactors are present and phosphorylated in germinating barley embryos. Therefore, we set up a large scale 14-3-3 affinity purification, to identify 14-3-3 interactors in embryos of barley seeds. Barley seeds are relatively large (Ø 7-9 mm), what makes it is possible to dissect the embryo and prepare protein extracts from this specific tissue. This kind of experiment can not be performed using, for example, the plant model *Arabidopsis*, with seed diameters of 0.5-1 mm. In this study, we identified twelve 14-3-3 interacting proteins from germinating barley embryos of which two turned out to be novel interactors, viz. RuBisCO activase and nucleolysin TIAR protein.

RESULTS

Functionality of Hv14-3-3 proteins and peptide competition

To purify 14-3-3 protein interactors from barley embryos we used an affinity purification approach using recombinant barley 14-3-3 isoforms, as described by (Moorhead et al., 1999). We prepared hexa-histidine tagged recombinant proteins of all five barley 14-3-3 isoforms (Figure 1). To test whether the recombinant proteins are biologically active, we used nitrate reductase (NR) as reporter system. NR is one of the best characterized 14-3-3 protein interactors in plants (Bachmann et al., 1996a; Moorhead et al., 1996; Ikeda et al., 2000; Athwal and Huber, 2002; Sinnige et al., 2005a). The activity of NR is inhibited by 14-3-3 proteins when NR is phosphorylated in the dark. NR assays were performed in the presence of different concentrations of recombinant Hv14-3-3C. We chose this isoform since it is the most efficient inhibitor of NR, as shown by Sinnige et al., (2005a).

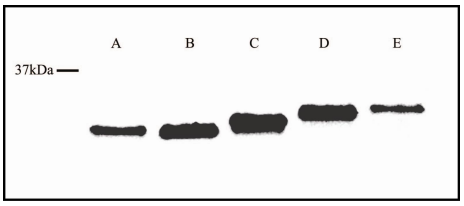

Figure 1. Recombinant protein of the five barley 14-3-3 isoforms

Recombinant HIS tagged protein was prepared of all five barley 14-3-3 isoforms. Lanes are marked according to the isoform. Two µg of each isoform was run on 12% SDS-PAGE gel and stained with colloidal Coomassie (BIO-RAD).

As shown in figure 2A, the recombinant 14-3-3C protein is a potent inhibitor of the barley NR enzyme. The Hv14-3-3C protein inhibits the barley NR enzyme with a K_i of 1.39 nM (Hv14-3-3C; MW: 33.8 kDa). The specificity of the affinity purification method is greatly increased by using a competing peptide that has high affinity for 14-3-3 proteins as shown by (Moorhead et al., 1999). At 0.5 mM, the peptide specifically elutes bound target proteins from the 14-3-3 protein column. In this study we have used the non-phosphorylated peptide R18, which was identified in a phage display experiment (Wang et al., 1999); the affinity of the R18 peptide for 14-3-3 binding was shown to be 80 nM. To test whether the R18 peptide also binds the barley recombinant 14-3-3C protein with high affinity, we used the inhibition of NR by 14-3-3 proteins as reporter system. As shown in figure 2B, the R18 peptide competes with the NR enzyme for the Hv14-3-3C proteins with a K_i of 0.39 µM. Therefore, we conclude that also for barley, the R18 peptide is a good tool for the elution of target proteins that are bound to a 14-3-3 column.

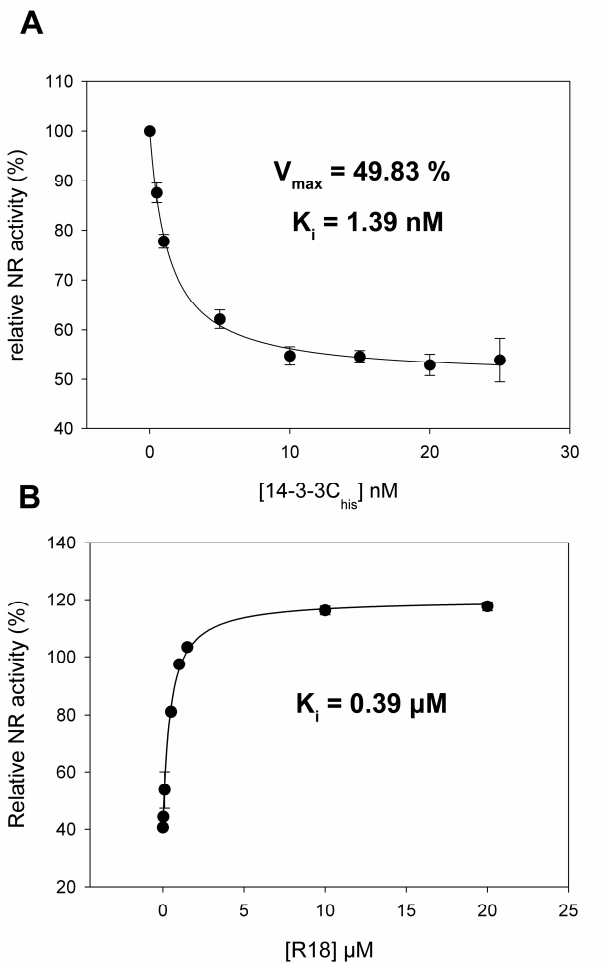

Figure 2. Quality control of recombinant barley 14-3-3C protein

(A) Functionality of the recombinant Hv14-3-3C protein was tested using the inhibitory action of 14-3-3 proteins on nitrate reductase. Nitrate reductase was semi-purified from barley leaf extract. The affinity of Hv14-3-3C for nitrate reductase was tested using different concentrations of recombinant Hv14-3-3C, ranging from 1 to 25 nM (MW 14-3-3C = 29.9 kDa). **(B)** Functionality of the competing peptide R18 was tested as its effectiveness to counteract the inhibitory action of 10 nM Hv14-3-3C inhibition on the Nitrate Reductase enzyme. Concentration range of the R18 peptide ranged from 10 nM to 20 µM.

14-3-3 binding partners in germinating barley embryos

Germinating barley embryos were chosen to search for new 14-3-3 protein interactors. Due to the size of the barley grain it is quite easy to isolate large amounts of embryonic proteins; this kind of study would never have been possible with *Arabidopsis* seeds. Forty grams of embryos were isolated and protein extracts were prepared as described in the materials and methods. In our lab, we have experienced that when 14-3-3 affinity purifications were performed on crude extract we did not detect any 14-3-3 interactors. However, when the protocol of Moorhead et al., (1999) was used, which includes an ammonium sulfate precipitation step, followed by tube dialysis, we successfully isolated 14-3-3 interactors. This suggests that an unknown factor in the crude extract interferes with the 14-3-3 target binding. As mentioned above, the specificity of this method is greatly enhanced by elution with the 14-3-3 competing peptide R18. As control, a mock elution was performed prior to the R18 elution, using the same procedure and the same buffer as with the R18 elution. As shown in Figure 3A, the mock elution shows some leakage of the recombinant 14-3-3 proteins (≈ 35 kDa). The R18 elution shows several bands between 50 and 75 kDa; two higher bands, one around 150 kDa and another around 250 kDa, were visible on Biosafe coomassie (BIO-RAD). To check whether 14-3-3 proteins bind to these individual proteins or that the proteins were part of a large complex, we performed far-western analysis (Figure 3B). All proteins that were visible on coomassie showed an interaction with the Hv14-3-3Cbio in the far-western experiment. To demonstrate that the far-western analysis is specific, the recombinant biotinylated 14-3-3C proteins were incubated together with an excess of R18 peptide. As shown in Figure 3B, the R18 peptide effectively blocked the interaction between the 14-3-3 proteins and the proteins on the membrane. From this result we conclude that the proteins that are visible on coomassie, bind directly to the 14-3-3 proteins and thus are not part of a complex that indirectly binds 14-3-3 proteins.

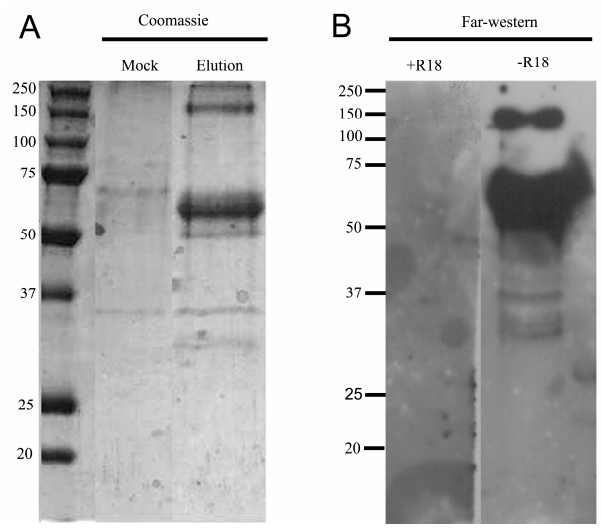

Figure 3. SDS-PAGE separation of 14-3-3 affinity purified targets

(A) The 14-3-3 affinity purified proteins from the barley embryo protein extract were specifically eluted using 0.5 mM R18 peptide. Prior to the 'specific' elution, a mock elution was performed using the same buffer composition, volume, and tubing. Both elutions were run out on a 12 % SDS-PAGE gel and proteins were stained with BIO-RAD Biosafe colloidal coomassie. **(B)** Proteins from the R18 elution were separated on a 12% SDS-PAGE gel and were transferred to a PVDF membrane. Far-western analysis was performed by incubating the membranes with 2µg/ml biotinylated Hv14-3-3C in the presence (+R18) and absence (-R18) of 100 µM R18 peptide. Membranes were washed extensively and after 2 hours membranes were incubated with avidin conjugated HRP. Subsequently, Amersham ECL luminescence was used to visualize the biotinylated Hv14-3-3C.

Identification of 14-3-3 target proteins

Both, the mock and the R18 elutions were analyzed using nano LC-ESI-MS/MS. The database search of the spectral data resulted in twelve matching proteins (Table 1). The majority of the proteins identified in this screen were recently identified in a 14-3-3 affinity purification experiment that we performed using seven days old barley seedlings (Chapter 6). Five proteins are metabolism related, of which four are invertase like proteins, six are signaling related, and one plays a role in protein translation (Table 1). From the twelve identified proteins present in the R18 elution, one protein was also identified in the mock elution, viz. RuBisCO large subunit. However, as can be observed in Figure 3A, the intensity of the 53 kDa band is much stronger in the R18 elution as compared to the mock. RuBisCO is known to be an abundant protein in leaf tissue and is often referred to as 'the most abundant' protein on earth. We speculate that RuBisCO is identified in the mock

elution since the recombinant 14-3-3 proteins leaked off the Ni^{2+}-NTA column. Therefore, targets, of which RuBisCO is the most abundant one, are identified in the mock elution as well. Two of the twelve proteins are novel 14-3-3 interactors viz. RuBisCO activase and the nucleolysin TIAR protein.

DISCUSSION

Recently, our group has identified the barley 14-3-3 protein family as an important signal mediator in the ABA and GA signal transduction pathways in germinating barley seeds (Chapter 2 and 3). In this study we capitalized on a 14-3-3 affinity purification method to identify novel 14-3-3 interactors in germinating barley embryos.

Initially, we set up a small scale purification system using avidin coated beads, decorated with biotinylated recombinant 14-3-3 proteins. These beads are suitable for incubation with small volumes of protein extracts. Rapid buffer changes characteristic for this method make it possible to use extracts derived from a variety of different developmental stages, tissues, and differently treated barley plants. Unfortunately, the small scale 14-3-3 affinity purifications were not very effective and targets were only identified when 14-3-3 interacting proteins were pre-purified, on for example anion exchange columns. However, when a complex mixture of proteins is used, this method does not result in the identification of novel 14-3-3 interactors (Sinnige et al. unpublished data). Many novel 14-3-3 interactors have been identified by using large scale14-3-3 affinity purification systems in a variety of different organisms (Moorhead et al., 1999; Pozuelo Rubio et al., 2004; Alexander and Morris, 2006; Angrand et al., 2006). The large scale affinity purification system is, just like the small scale purification system, inefficient but due to the high amount of proteins used, it does result in the identification of 14-3-3 interactors. We estimate that if every eluted protein directly binds one 14-3-3 dimer, in a ratio of 1:1, the 14-3-3 to target ratio in our experiments is approximately 1:1000.

To test whether the recombinant 14-3-3 proteins are functionally active we used the well characterized 14-3-3 interactor, nitrate reductase (NR). NR is known to be phosphorylated in the dark and subsequently inhibited by 14-3-3 proteins. Here, we semi-purified NR from plants that were held in the dark for 30 minutes, and subsequently tested its activity in the absence and presence of recombinant 14-3-3C protein. The inhibition of NR by 14-3-3C is comparable to our previous studies (K_m = 1.39 µM) and thus suggests that the recombinant proteins are intact and properly folded as shown by Sinnige et al., (2005) using Circular Dichroism (CD) analysis.

The 14-3-3 affinity purification from germinating barley embryos resulted in twelve 14-3-3 interactors. Comparing the list of identified proteins with the coomassie stained 1D SDS-PAGE gel (Figure 3A), we conclude that not all proteins that are visible on the coomassie stained gel are identified in the ESI-MS/MS analysis. The molecular mass of the identified proteins ranged from 20 to 55 kDa (Table 1). In contrast, the proteins separated on the SDS-PAGE gel ranged from 20 to 250 kDa (Figure 3). We hypothesize that the larger proteins (> 55 kDa) were not identified because of the low protein yield. Coomassie staining of high molecular mass protein results in higher signals per mole protein compared to the low molecular mass proteins. Moreover, in gel digestion of high molecular mass proteins is known to be less efficient and also results in a more difficult identification of proteins.

Two novel 14-3-3 interactors were identified in this study, namely RuBisCO activase and nucleolysin TIAR protein. The latter protein is a homologue of Fava bean AKIP1, human TIAR, and rice UBP1 interacting protein 1A (UBA2a). The protein contains two RNA recognition motifs (RRMs) and is capable of binding RNA. VfAKIP1 was shown to be a substrate for the abscisic acid-activated-protein-kinase (AAPK) and phosphorylation of the AKIP1 is required for RNA binding to the ABA inducible dehydrin mRNA (Li et al., 2002). However, neither gain-of-function nor loss-of-function mutants of the *Arabidopsis* homolog UBA2a showed the expected ABA phenotype (Riera et al., 2006). Further research has to reveal whether 14-3-3 interaction with the phosphorylated TIAR protein plays an important role in the interaction between TIAR and RNA molecules.

RuBisCO is the first enzyme in the reductive pentose phosphate (Calvin) cycle, which catalyzes the reaction of ribulose bisphosphate to 3-phosphoglycerate. Recently, an additional function for RuBisCO was shown, Schwender et al., (2005) showed that RuBisCO plays a role in tissue lacking Calvin cycle activity, where it increases the efficiency of oil production and decreases carbon loss (Schwender et al., 2004). The activation of RuBisCO occurs when RuBisCO is carbamylated at E^{201} and subsequently binds Mg^{2+}. Sugar phosphates bind to the same site, and negatively regulate RuBisCO by preventing the binding of Mg^{2+} ions. RuBisCO activase prevents binding of sugar phosphates to the catalytic site of RuBisCO, what results in the activation of RuBisCO (von Caemmerer et al., 2005). RuBisCO activase has been shown to form a complex with RuBisCO large subunit, carbonic anhydrase, phosphoribulokinase, and 3-P-glycerate kinase (Suss et al., 1993; Anderson and Carol, 2004). Suss et al. (1993) showed that this multi-enzyme complex is bound to the chloroplast thylakoid membrane. Nevertheless, chloroplasts are not present in the material used in this study and therefore we hypothesize

Table 1. Identified protein from the R18 elution

Mass spectral data were searched against two plant databases. Accession numbers are presented for different databases where the superscript letters denotes: A: NCBI-viridiplants and B: ZMGI.

#	Protein name	Accesion number	Mw (kDa)	Mowse score (Peptides matched MS/MS)	Gel ID	Function	Reported	14-3-3 motif
1	Beta-fructofuranosidase	AT1G22650.1[A]	52.21	344 (6)	1	Metabolism	Yes	-----
2	Ribulose bisphosphate carboxylase/oxygenase activase / RuBisCO activase	AT2G39730.1[A]	51.97	63 (1)	1	Signaling	No	KNFLTLP
3	RuBisCO large chain	ATCG00490.1[A]	52.95	51 (1)	1	Metabolism	Yes	-----
3	Neutral invertase like protein	DR828403[A]	EST	63 (1)	1	Metabolism	Yes	-----
4	Alkaline/ neutral Invertase	TC290700[B]	26.32	72 (2)	1	Metabolism	Yes	-----
5	14-3-3-like protein GF14-6	TC298517[B]	29.66	142 (2)	1	Signaling	Yes	-----
6	Invertase	TC301560[B]	29.78	201 (3)	1	Metabolism	Yes	-----
7	Eelongation factor 1-alpha	AT1G07920.1[A]	49.49	61 (1)	2	Translation	Yes	KMDATTP
8	14-3-3 protein GF14 iota (GRF12)	AT1G26480.1[A]	30.54	35 (1)	2	Signaling	Yes	-----
9	Nucleolysin TIAR (TIA-1 related protein)	TC280532[B]	41.59	40 (1)	2	Signaling	No	-----
10	GF14-d protein	TC279949[B]	29.26	231 (6)	3	Signaling	Yes	-----
11	Putative acid phosphatase	TC302171[B]	29.60	50 (1)	3	Signaling	Yes	RTFSRP

that the interaction between 14-3-3 and RuBisCO plays a role in increasing the carbon fixation during oil synthesis (Schwender et al., 2004). Interestingly, many proteins that belong to this multi-enzyme complex have recently been shown to interact with 14-3-3 proteins (Table 1 and Chapter 6). Since most of these proteins were identified in different experiments it is suggested that plasticity exists for this multi-enzyme complex and we hypothesize that 14-3-3 proteins form the adapter protein to bring the right proteins together into the complex.

Comparing the results of the 14-3-3 affinity purification from this study with other studies, e.g. (Moorhead et al., 1999; Milne et al., 2002; Pozuelo Rubio et al., 2004; Alexander and Morris, 2006), we observe that the affinity purification method is inefficient. As mentioned in the results, just one in every thousand 14-3-3 dimers binds a target protein. Therefore, large amounts of proteins have to be applied to a 14-3-3 column. Studying the 14-3-3 interactome in embryos is due to this inefficiency not an easy task, even for barley with its large seeds. The proteins identified in this study are; invertase like proteins, elongation factor 1A, acid phosphatase, 14-3-3 proteins, and RuBisCO. These proteins all have in common, that they are highly abundant proteins. These data indicate that the lower abundant proteins, most likely bind to the 14-3-3 column, but are not detectable on coomassie and nano-LC-MS/MS. Moreover, using barley to perform 14-3-3 affinity purification has the disadvantage that the genome has not been sequenced yet and thus many peptides that are found by MS/MS analysis, do not correspond to peptides in the database, due to lack of homology with other proteins.

MATERIALS AND METHODS

Plant Material

Seven days old barley seedlings (*Hordeum vulgare* cv Alexis) were used for the NR experiments and the seeds were a kind gift of Josef Breun Saatsucht (Herzogenaurach, Germany). For the 14-3-3 affinity purification, barley (*Hordeum vulgare* cv Himalaya) seeds were used (harvest 1998, Pullman Crop and Soil Sciences, Washington State University).

Preparation of constructs

The 14-3-3 genes were cloned from cDNA using PCR and subsequently ligated into the Invitrogen pGEM-T easy vector using T/A cloning, as described before for the isoforms A, B, and C (Sinnige et al., 2005a). Primers were designed on the 14-3-3 genes introducing BamHI sites at both sites and amplified 14-3-3 genes were subsequently cloned in frame

into the Invitrogen pRSET-C vector. The ORF of the 14-3-3 genes were checked by performing sequencing reactions using the Big Dye sequencing kit (Applied Biosystems).

Preparation of recombinant proteins

Recombinant proteins were prepared by electroporation of the appropriate DNA construct into BL21 E.coli cells. BL21 cells containing the construct were grown in YT medium for 16 hours at 37 °C, then Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was grown for another 8 hours and subsequently *E. coli* cells were spun down and recombinant proteins were isolated using the Amersham His-trap column (1 ml) according to the manufacturer's protocol. The quality of the recombinant proteins was checked by performing SDS-PAGE and western blotting using the Novagen T7-tag antibody.

Purification of NR from barley leaf extract

Purification of NR was performed as described previously (Sinnige et al., 2005a). Briefly, 7 days old barley plants were put in the dark for 30 minutes. Subsequently, 5 grams of leaves were ground in liquid nitrogen and 10 ml of homogenization buffer (100 mM Hepes-KOH pH 7.5, 20 mM NaF, 10 mM EDTA, 20 µM FAD, 5 µM $NaMoO_4$, 0.1 % (w/v) Triton X-100, 6 mM DTT, 10 µM Cantharidin, Roche Complete Protease Inhibitor Cocktail (3 tablets per 500 ml), 1 mM PMSF, 1 % (w/v) PVPP) was added. Homogenate was centrifuged for 30 minutes at 100,000*g* and supernatant was filtered through 0.2 µm cellulose-acetate filters (Schleicher & Schuell MicroScience). The filtrate was then run through a 5 ml HiTrapQ (Amersham) anion-exchange column, the homogenization buffer without PVPP was used as running buffer and proteins were eluted from the column with a 30 min linear salt gradient using 0.5 M NaCl as final salt concentration. During the salt gradient, fractions of 1.5 ml were taken. Each of the fractions was tested for NR activity and fractions with highest activity were pooled.

Protein extraction and 14-3-3 affinity purification

Barley cv Himalaya seeds were imbibed in water and germinated for 3 days in the dark at 20 °C. Subsequently, 40 grams of fresh weight embryos were dissected and homogenized in buffer A (50 mM Hepes-KOH pH 7.5, 50 mM NaF, 5 mM NaPPi, 1 mM DTT, 1 mM PMSF, Roche Complete Protease Inhibitor Cocktail (1 tablet per 50 ml), 1 % (w/v) PVPP). The 14-3-3 affinity purifications were performed as described by Moorhead et al., (1999),

except that we used a total of 5 mg of recombinant 14-3-3 proteins in a volume of 15 ml protein extract with a protein concentration of 8 mg/ml (± 0.12 g of total protein).

Far-western analysis

Far western analysis was performed according to Moorhead et al. (1999). Briefly, proteins were separated by performing SDS-PAGE and subsequently transferred to Immun Blot™ PVDF (BIO-RAD) membrane using BIO-RAD semi-dry blotting system. Blots were incubated with buffer A (25 mM Tris-HCl pH 7.5, 500 mM NaCl) containing 5 % milk powder (Marvel) for 16 hours. Subsequently, blots were washed 3 times with Buffer A and then incubated with buffer A containing 1 µg /ml BSA and 3 µg /ml recombinant biotinylated 14-3-3 proteins for 2 hours. Blots were washed extensively with buffer A for 2 hours, refreshing the buffer every 15 minutes. The bound 14-3-3 proteins were visualized using avidin-HRP conjugated antibody and the Amersham ECL kit using the manufactures' protocol.

Mass spectrometry

The mock and the R18 elutions were run on a 12% 1D SDS-PAGE gel. To identify the proteins, the lanes were cut from top to bottom of the gel in four pieces. In gel digestion was performed as described previously (Friso et al., 2004), and peptides were eluted from the gel pieces. The peptides were separated and identified using nano liquid chromatography – electron spray ionization - tandem mass spectrometry (nano LC-ESI-MS/MS) analysis using a Quadrupole / time of flight (Q-TOF) mass spectrometer (Waters). Spectral data were extracted using Mascot distiller and searched with Mascot (Matrix Science) against NCBI nr - viridiplants, rice (OsGI - v4 from Tigr at http://www.tigr.org/), maize (ZmGI - v6; Tigr) and *Arabidopsis thaliana* (Tair v6 at http://www.arabidopsis.org/).

Chapter 6

A comprehensive analysis of the 14-3-3 interactome in barley leaves using a complementary proteomics and two-hybrid approach

With: Helena Veiga, Daniel da Costa Pereira, Giulia Friso, Klaas J. van Wijk, and Albertus H. de Boer

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ABSTRACT

This study describes the identification of over 150 target proteins of the five 14-3-3 isoforms in 7 days old barley (*Hordeum vulgare* cv. Himalaya) seedlings using yeast two-hybrid screens complemented with 14-3-3 protein affinity purification and tandem mass spectrometry. Independent experiments for a subset of genes confirmed the yeast two-hybrid interactions, demonstrating a low false positive identification rate. These combined approaches resulted in the identification of more than 150 putative targets; 15 % were previously reported to be 14-3-3 interactors, including e.g. Serpin, RF2A, WPK4 kinase, P-type H^+-ATPase, EF1A, glutamine synthetase, and an invertase. The affinity purification resulted in 30 interactors of which 44 % function in metabolism, while the yeast two hybrid screens identified 132 different proteins with 35 % of the proteins involved in signal transduction. A number of proteins have a well described function in hormonal signaling, such as the auxin transport protein PIN1 and NPH3 and components of the brassinosteroid pathway, such as the receptor kinase BAK1 (OsPERK1) and BRI1-KD interacting protein 129 (BIP129). In addition, three ABF-like transcription factors and a RSG orthologue named RF2A were identified; these transcription factors play important roles in the ABA and GA pathways, respectively. We speculate that 14-3-3 proteins have a role in cross-talk between these hormonal pathways. The specificity and complementary nature of both the affinity purification and the yeast two-hybrid approaches is discussed.

INTRODUCTION

Phosphorylation dependent protein-protein interactions play crucial roles in the execution of various biological functions (Mukherji, 2005). Most widespread amongst proteins that interact with phosphoserine or phosphothreonine motifs are members of the 14-3-3 family (Van Heusden, 2005). 14-3-3 proteins are acidic regulatory proteins, first identified as highly abundant proteins in bovine brain extract (Moore and Perez, 1967). Later it was found that 14-3-3 proteins are present in all eukaryotes (de Vetten et al., 1992; Hirsch et al., 1992; van Heusden et al., 1992). 14-3-3 proteins belong to a conserved family consisting of 7 isoforms in human, 2 isoforms in yeast, 15 isoforms in *Arabidopsis*, and 8 isoforms in rice (van Heusden et al., 1995; Van Hemert, 2001; Sehnke et al., 2002; Chen et al., 2006).

The crystal structure of the 14-3-3 dimer revealed that it forms a clamp shape structure with dimerization occurring at the N-terminus. Co-crystallization of the 14-3-3 dimer together with the Raf phosphopeptide showed that the clamp shape structure has two binding grooves that both can bind a peptide (Yaffe et al., 1997). Therefore, one 14-3-3 dimer can bind two target proteins at the same time or can bind one target protein at two specific sites (Braselmann and McCormick, 1995). (Muslin et al., 1996) showed that binding sites have a conserved amino acid composition that belongs either to the mode I (R/K-S-X-$_p$S/T-X-P) or mode II (R/K-X-Y/F-X-$_p$S/T-X-P) type. Binding to the target protein depends on the phosphorylation of the serine or threonine residues inside the binding site in most, but not all cases.

14-3-3 proteins are known to regulate a variety of different cellular processes, such as cell division, apoptosis, signaling, and carbon and nitrogen metabolism (Van Hemert, 2001; Huber et al., 2002; Schoonheim et al., 2007). Most 14-3-3 targets identified to date in plants are metabolism related enzymes, such as: glutamine synthetase (GS), nitrate reductase (NR), glyceraldehyde phosphate dehydrogenase (GAPDH), sucrose phosphate synthase (SPS), trehalose phosphate synthase (TPS), and invertase (Moorhead et al., 1996; Moorhead et al., 1999; Huber et al., 2002). In the study of (Pozuelo Rubio et al., 2004) more than 200 14-3-3 interacting proteins in human cancer cells were identified using affinity chromatography. In this study it was pointed out that there seems to be a large functional difference in the identified 14-3-3 targets in animal cells as compared to plant cells. For example, the number of plant 14-3-3 targets functioning in signal perception (receptors), transduction (kinases) and processing (transcription factors) is small as compared to animal cells. This difference is surprising in the light of the conserved

nature of 14-3-3 proteins. We hypothesize that the current picture of the plant 14-3-3 interactome is biased due to the methods used so far to identify 14-3-3 target proteins. Therefore, the aim of this study was to carry out a comprehensive identification of 14-3-3 targets present in barley leaf tissue using two complementary methods: a yeast two-hybrid screen and an affinity purification strategy using all five known barley 14-3-3 proteins.

Yeast two-hybrid screens have been used to identify protein-protein interactions extensively (Fields and Song, 1989; McAlister-Henn et al., 1999; Ito et al., 2001). In the field of 14-3-3 research yeast two-hybrid assays have been widely used to confirm and analyze interactions between one 14-3-3 protein and a specific target (Bornke, 2005). Large scale yeast two-hybrid screens of cDNA libraries using 14-3-3 as bait have been carried out as well, although the number of interacting proteins reported was rather low (Zhang et al., 1997; Yan et al., 2002; Bornke, 2005; Mackie and Aitken, 2005). An important advantage of the yeast two-hybrid screen is that large amounts of cDNA clones can be checked with relative ease and in a short time. A second, and may be even more important advantage of this method is that low-abundant transcripts isolated from plant tissue stand a good chance to be co-expressed with the 14-3-3- GAL4 binding domain, and therefore its protein product can be identified as 14-3-3 targets. This is because cDNA clones are under the control of the constitutive *ADH1* promoter and are therefore highly expressed in the yeast. This exact same feature of the yeast two-hybrid method can also be interpreted as a disadvantage, namely the identification of false positives. The high concentration of proteins present in the yeast cells might result in interactions that will not occur in the plant cell. Nevertheless, the yeast two-hybrid method is a good tool to study the putative interaction between two proteins, which subsequently have to be studied in more detail *in vivo* by for example performing far-western or FRET-FLIM analysis.

The second method to study protein-protein interactions, also extensively used in the field of 14-3-3 biology, is affinity chromatography with 14-3-3 as bait, followed by identification using mass spectrometry (Moorhead et al., 1999; Pozuelo Rubio et al., 2003; Pozuelo Rubio et al., 2004; Alexander and Morris, 2006). Due to the dynamic nature of the 14-3-3/target interaction, it is possible to selectively compete off target proteins with a peptide such as the so-called R18 peptide mimicking a phosphorylated mode I or mode II motif (Moorhead et al., 1999; Wang et al., 1999). In this way false positives are to a large extent eliminated. Moreover, other than in a yeast two-hybrid screen, affinity chromatography can purify protein complexes when a 14-3-3 target protein is part of that complex. This can provide more comprehensive insight into protein networks involving 14-

3-3 proteins but has as disadvantage that it is not clear whether the protein binds indirect or directly to the 14-3-3 proteins.

In this study we show that the yeast two-hybrid screen is an effective tool to identify putative 14-3-3 targets. We identified 132 proteins that interact with at least one of the five barley 14-3-3 isoforms. The affinity chromatography approach yielded thirty 14-3-3 target proteins with the majority having a function in primary metabolism, possibly reflecting a bias of this method to more abundant proteins. Most of the proteins identified in the two-hybrid screen are signal mediators, providing evidence that plants 14-3-3 proteins do not only play an important role in regulation of the Calvin cycle, glycolysis, and nitrogen metabolism, but are also important intermediates in signaling cascades. Combining published targets and novel targets identified in this study, an interaction map for the plant 14-3-3 proteins is emerging.

RESULTS

Novel interaction partners for barley 14-3-3 proteins identified in a yeast two-hybrid screen

To identify proteins which interact with each of the five known barley 14-3-3 isoforms (A, B, C, D and E), we took advantage of the sensitive yeast two-hybrid protein-protein interaction assay to screen a barley leaf cDNA library (Robertson, 2004). Clearly, plant 14-3-3 proteins are functionally and properly folded in yeast, since they are able to complement the lethal yeast double knock-out mutants for *BMH1* and *BMH2* (van Heusden et al., 1995). Since 14-3-3 proteins can form homo- and heterodimers, we first addressed the question whether positive clones might show up through dimerization of one 14-3-3 in the activation domain (14-3-3-AD) and another (or the same) 14-3-3 in the binding domain (14-3-3-BD). A yeast two-hybrid assay between the 14-3-3 isoforms showed weak homo-dimerization of 14-3-3B, whereas the other 14-3-3 isoforms showed no homo- or hetero-dimerization (data not shown). These data show that dimerization of the 14-3-3 isoforms would not overwhelm us with the identification of a majority of 14-3-3-AD fusions. Next, we tested whether auto-activation of one of the reporter genes might occur. When the 14-3-3 proteins were fused to the GAL4 binding domain (14-3-3-BD) and double yeast transformants of the 14-3-3-BD vs. empty-AD were tested on the activation of the three reporter genes viz. *HIS3, ADE2,* and *LacZ*, indeed auto-activation of the *HIS3* reporter gene was observed; therefore this reporter was not used in the two-hybrid screen. In contrast, no auto-activation of the the other two reporter genes was measured.

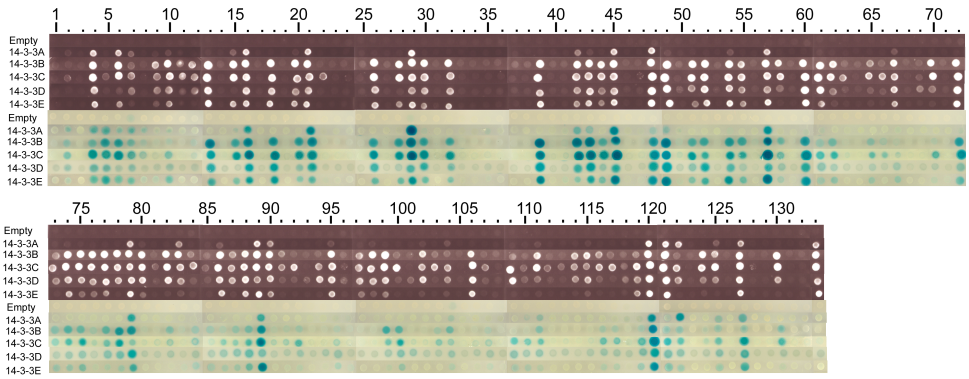

Figure 1. Yeast two-hybrid assays between five barley 14-3-3 isoforms and the 132 identified 14-3-3 interactors

All the 132 unique 14-3-3 interactors identified in the yeast two-hybrid screens were re-transformed into yeast carrying one of the barley 14-3-3 isoforms and are numbered from spot 2 to 133. Interaction is confirmed by growing the double-transformants on selective media (top panel) (SD minus LWHA) or performing beta-galactosidase assay (lower panel). As control, empty-BD constructs were used against the 14-3-3 interactors (top lane), and empty-AD against the 14-3-3-BD (spot 1).

Thus, for the two-hybrid screens a total of 1.7 x 10^6 double transformants were screened for activation of the reporter genes *ADE* and *LacZ*. Each 14-3-3 isoform was used in a separate screen and in these five screens a total of 132 unique proteins were identified as positive clones for both reporter genes, *ADE* and *LacZ*, and thus putative new 14-3-3 target proteins (Table 1). All positive clones (132) were checked for the presence of an “in frame” cDNA-AD fusion. The clones were then re-transformed into yeast containing the 14-3-3 bait to confirm their interaction on selective plates (SD minus LWHA) in combination with the presence of β-galactosidase activity (Figure 1).

Isoform specificity in 14-3-3/target interaction is an important issue in 14-3-3 biology (Bornke, 2005; Sinnige et al., 2005a), and therefore we tried to evaluate differences in the strength of interaction between each putative target and each individual 14-3-3 isoform. In order to create more stringent conditions, diluted cultures were spotted on selective plates (SD minus LWHA) and grown for only 1 day. As a consequence, some weaker interacting clones did not grow at all under these conditions with any of the 14-3-3 proteins and did not turn blue in the β-galactosidase assay (Figure 1). However, it should be noted that these clones did grow with at least one of the five 14-3-3 isoforms when undiluted cultures were spotted and turned blue when longer incubation times were used (data not shown). Therefore, all 132 clones have to be interpreted as putative 14-3-3

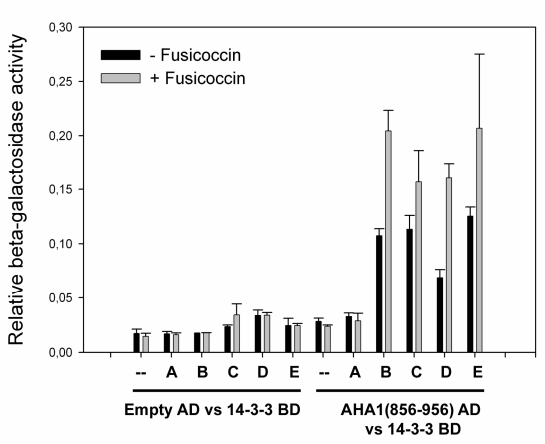

Figure 2. 14-3-3 interactions with the P-type H^+-ATPase is induced by Fusicoccin

Beta-galactosidase assays of double-transformants of the 14-3-3 isoforms together with the C-terminal tail of the P-type H^+-ATPase show that fusicoccin enhances beta-galactosidase activity of double transformants. FC (10 µM) treatments were given for 24 hours and subsequently beta-galactosidase assays were performed (n=3 ± SD). Beta-galactosidase activities are normalized to the OD600. Fusicoccin treatment did not have an effect on yeast growth.

targets even though there is little or no growth in Figure 1. The first column in Figure 1 show that none of the 14-3-3 proteins are auto-activators of the used reporter genes, since double-transformants of 14-3-3-BD and empty-AD (spot 1) shows no growth and no blue color on beta-galactosidase assay. Vice-versa, none of the interacting proteins showed auto-activation when transformed with an empty-BD vector (Figure 1; first row). Importantly, clear isoform specific interactions were observed: some targets show high affinity for all five 14-3-3 proteins (Figure 1; spots 21, 29, 57, 79, 89 and 120), whereas others interact with only one or two isoforms (Figure 1; spots 22, 63, 66, 84, 92, 100, 117).

Identification of isolated clones and validation

About 10% of the identified genes represent previously identified and characterized 14-3-3 targets. The presence of these known 14-3-3 targets in our screen can be considered as positive control. One of the best characterized 14-3-3 targets was identified amongst these positive controls, namely the P-type H^+-ATPase (Figure 1; spot 54). The cDNA that was identified consists of the soluble C-terminal cytosolic auto-inhibitory domain; this part is not membrane bound, thus explaining its presence in the yeast two-hybrid screen. The cDNA encodes for the last C-terminal 101 amino acids including the 14-3-3 binding site, YTV-COOH.

It is well known that the phytotoxin fusicoccin (FC), stabilizes the complex formed by 14-3-3 proteins and P-type H^+-ATPase and increases the affinity between these two proteins (Fuglsang et al., 1999). We used the 14-3-3/ATPase interaction to demonstrate that our two-hybrid assay is capable of detecting small differences in strength of interaction between 14-3-3 and a target. So, addition of FC (10 µM) to yeast cells containing the 14-3-3 proteins as bait and the C-terminus of the P-type H^+-ATPase as prey, should enhance the β-galactosidase activity. Figure 2 shows that FC addition indeed results in a 1.5 to 2 fold induction of the *LacZ* reporter gene. This result fits with the literature and suggests that the interaction measured with the yeast two-hybrid correlates with the interactions that occur *in planta*. Moreover, the yeast two-hybrid principle is potentially a good method to screen for novel FC receptors.

It is beyond the scope of this work to validate all 132 putative 14-3-3 targets and therefore we focused on three members of the AREB/ABF/ABI5 family (HvABF1, HvABF2, and HvABF3) that we isolated in this screen (Figure 1; spots 13, 20 and 49). Most members of the ABF/AREB/ABI5 protein family contain a canonical mode II 14-3-3 interaction motif (RRTLT350GPW-COOH) that is also conserved in the related HvABI5 protein. Mutation of T^{350} in the HvABI5 protein, showed that this residue is essential for 14-3-3 interaction (Schoonheim et al., 2007). Here we show that deletion of the conserved C-terminal putative 14-3-3 binding motif in the ABF transcription factors (ABF-C8Del) disrupts the interaction with the 14-3-3 proteins in a yeast two-hybrid assay (Figure 3A and B). Moreover, we confirmed the interaction between the ABF proteins and 14-3-3C by performing far-western analysis using recombinant ABF proteins on blot and recombinant biotinylated Hv14-3-3C in the overlay buffer (Figure 3C). The GST-R18 peptide, known to bind 14-3-3 with high affinity, was included as positive control. Finally, 10 of the 132 identified proteins were also identified in the affinity chromatography experiment as shown below.

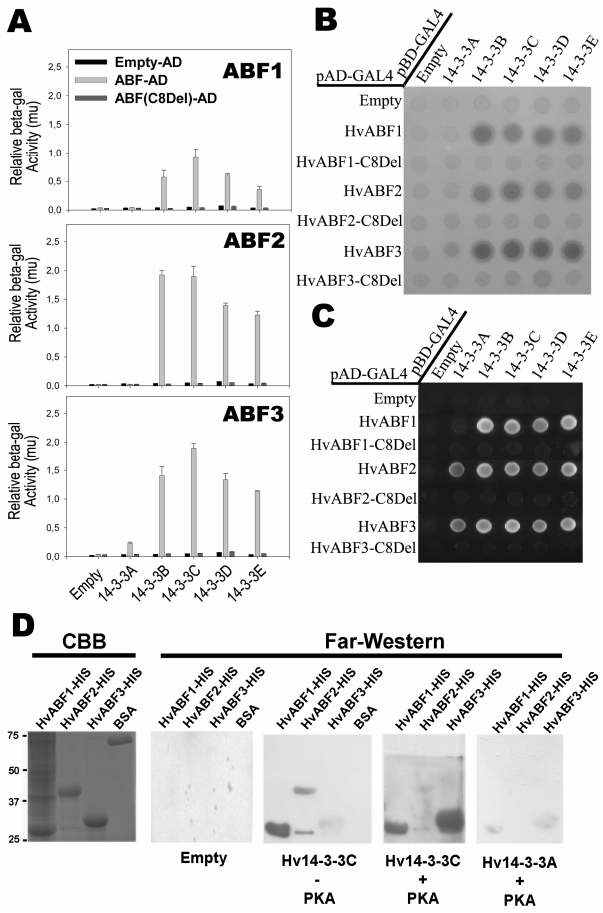

Figure 3. Three ABF transcription factors interact with 14-3-3 proteins

(A) Quantitative β-galactosidase activity of yeast cells carrying the 14-3-3 proteins as bait and the ABF transcription factors as prey. Eight amino acid deletion at the C terminus (C8Del) were tested for their interaction with the 14-3-3 proteins. **(B)** Semi-quantitative β-galactosidase activity assay of double transformed yeast cells carrying 14-3-3 proteins as bait and the ABF transcription factors (WT and C8Del) as prey. **(C)** Qualitative assays of the 14-3-3 interactions with the ABF transcription factors (WT and C8Del). **(D)** Interaction between recombinant protein of the transcription factors HvABF1, HvABF2, and HvABF3 and recombinant 14-3-3A and 14-3-3C is shown by performing far-western analysis. BSA was taken along as negative control. Protein Kinase A treatments was performed to phosphorylate the recombinant proteins.

14-3-3 proteins interact with a variety of proteins involved in signaling.

As discussed in the Introduction, the list of known 14-3-3 targets in plants contains far fewer proteins with a function in signal transduction than in animals. Table 1 and Figure 5A show that the main group of proteins identified in this yeast two-hybrid screen belongs to the class of signal mediators (35 %). Interestingly, many of these signal mediators belong to pathways of hormonal signal transduction. A few examples are:

- BRI1-KD (Kinase Domain) interacting protein 129 (BIP129) (spot 12), a protein that can be phosphorylated by BRI1-KD brassinosteroid receptor, and likely has a function in brassinosteroid signal transduction (Hirabayashi et al., 2004).
- The bZIP transcription factor family of the ABFs (spots 13, 20 & 49), which function in the abscisic acid pathway (Schoonheim et al., 2007).
- The bZIP transcription factor RF2A: the barley orthologue of the tobacco REPRESSION OF SHOOT GROWTH (RSG) that plays a role in the GA biosynthesis. RF2A shows strong interactions with all five barley 14-3-3 isoforms (spot 29).
- NPH3, a protein with a function in lateral auxin transport during phototropism (Haga et al., 2005) showing strong interaction with four out of five 14-3-3s as well (spot 48, Figure 1).

Hormone responsive transcripts were found as well: - the jasmonic acid responsive RRJ1 (TC140455) and jasmonate induced mRNA (TC131671) - for gibberellin, the gibberellin stimulated transcript1 (TC148126) and for auxin, Adr11 (TC147470).

14-3-3 Affinity purification and tandem mass spectrometry identifies novel 14-3-3 targets

In addition to the yeast two-hybrid screens we performed a large scale affinity chromatography experiment using recombinant proteins of all five barley 14-3-3 isoforms as bait. The five barley 14-3-3 proteins (A-E) were cloned into the Invitrogen pRSET vector resulting in N-terminal tagged 14-3-3- hexahistidine $(HIS)_6$ fusions. Recombinant proteins of all five 14-3-3 isoforms were produced in BL21-DE3 cells and purified using Ni^{2+}-NTA affinity chromatography. These $(His)_6$ tagged 14-3-3 isoforms were used as bait.

Our interaction analysis was based on two selective steps: i) specific binding of target proteins into the 14-3-3 groove and ii) selective elution of proteins that interact with 14-3-3 through this groove with a peptide that has a high affinity for the binding groove, called R18 (Wang et al., 1999). R18 is a peptide that specifically binds to 14-3-3 proteins without isoform selectivity. Scatchard analysis of R18 binding to 'empty' 14-3-3 showed K_d values in the range of 0.1 μM (Wang et al., 1999).

Protein extract from leaves of seven day old barley cv. Himalaya plants (identical to the source of the yeast two-hybrid cDNA library) was prepared. A total of 500 milligram of cytosolic proteins was mixed with 10 mg of recombinant His-tagged 14-3-3 in a total volume of 25 ml. After incubation at 4°C, the mixture was run through a Nickel column. After rinsing extensively, a control elution was performed with solely the injection of buffer. Next, bound target proteins were eluted from the column using 0.5 mM of the R18 peptide.

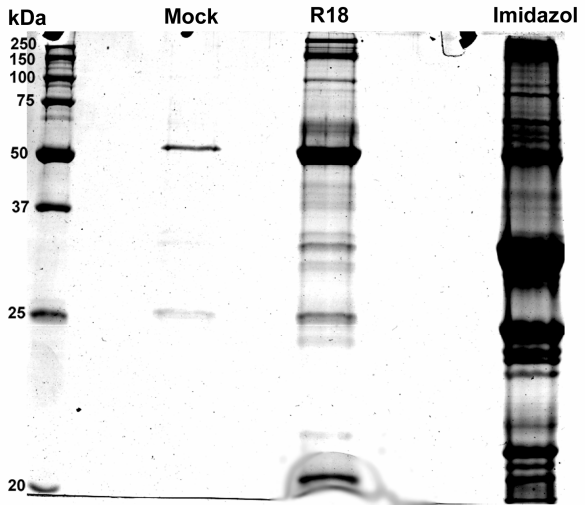

Figure 4. Elutions of 14-3-3 interactors

14-3-3 interacting proteins were specifically eluted using 0.5 mM R18 peptide. The mock elution, R18 elution, and the imidazol strip were concentrated and 10% of the samples were separated on a 12% SDS-PAGE gel. Proteins were stained with BIO-RAD Biosafe colloidal coomassie.

This relatively high R18 concentration (1250 times K_m) was chosen because the time of exposure is relatively short (flow rate 0.5 ml min^{-1}). Higher concentrations of R18 (1 mM) did not elute more bands (data not shown). Eluted proteins were separated on SDS-PAGE gel and the R18 elution resulted in many bands ranging from high molecular weight (250 kDa) to low molecular weight (15 kDa) on a colloidal Coomassie stained gel (Figure 4).

Mock and R18 eluate were run out on a 1D SDS-PAGE gel and the R18 lane was sliced in 6 pieces, proteins were in-gel digested with trypsin and peptides were identified using nano LC-ESI-MS/MS. The MS/MS data were searched against NCBI green plants and a recent version of the annotated rice genome (Tigr OsGI v4) and maize unigene assembly (Tigr ZmGI v16) using Mascot.

Identified proteins are listed in Table 2, ordered according to the gel slices ranging from high- to low molecular weight (gel slice 1-6). The M_r of proteins identified from the respective gel slices, correlates well with the predicted M_r range (based on the protein markers) of each gel slice that they originated from. Intriguingly, 45 % of the 30 eluted targets are metabolism related proteins and many of these are part of either glycolysis or the Calvin cycle (Table 2).

The two approaches that were used in this study identified a large group of putative 14-3-3 interactors that have to be studied in more detail in future studies. From the group of proteins identified using the 14-3-3 affinity purifications, 30% of the interacting proteins were confirmed in the yeast two-hybrid screen.

DISCUSSION

Classification of identified 14-3-3 targets

The yeast two-hybrid screens and the affinity purification show a large difference in the representation of different protein classes (Figure 5). More than one third (50) of the interacting proteins identified in the yeast two-hybrid screens fall in the class of signaling proteins; 17 of these are transcription factors. In contrast, only 10 % of the proteins identified in the affinity purification could be assigned a function in signaling and none of these were transcription factors. The 14-3-3 affinity purification method identified mostly metabolic enzymes (42 %). A similar observation was made by (Alexander and Morris, 2006), who purified 14-3-3 interacting proteins from developing barley grains using a 14-3-3 affinity chromatography strategy. Metabolic proteins are abundant proteins and are therefore probably preferentially identified when using 14-3-3 affinity chromatography. In contrast, signaling intermediates are low abundant proteins and are therefore less likely to be detected in the affinity purification.

Overlap between yeast two-hybrid and affinity purification

Although many differences can be found between the yeast two-hybrid screen and the affinity purification, also a large portion of the identified proteins overlap. 30 % of the identified proteins from the affinity purification were also identified in the yeast two-hybrid screens, like elongation factor 1A, neutral invertase, acyl-transferase, ATP-dependent Clp protease, RuBisCO small subunit, and 14-3-3 proteins themselves.

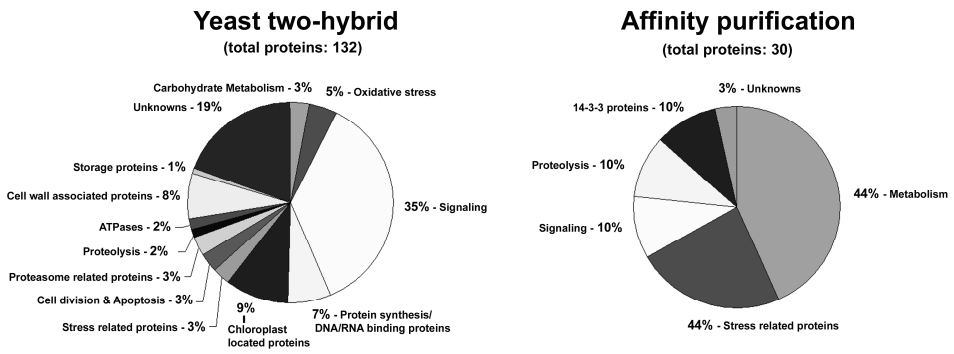

Figure 5. Classification of the 'novel' putative 14-3-3 interactors

The 132 identified proteins from the yeast two-hybrid screen were divided and classified into thirteen classes. The 30 proteins identified from the affinity purification were classified into six classes. Signaling associated proteins were found to be the major class of proteins identified in the yeast two-hybrid screens whereas metabolism related proteins were predominantly found in the 14-3-3 affinity purification.

In addition, several proteins only identified by affinity purification were reported before as 14-3-3 interacting proteins: ascorbate peroxidase, glutamine synthetase and GAPDH (Zhang et al., 1997; Moorhead et al., 1999; Cotelle et al., 2000). Likewise, many proteins previously reported in the literature as 14-3-3 interacting proteins were identified in the yeast two-hybrid screen: RF2A, serpin, WPK4 and the P-type H^+-ATPase (Ikeda et al., 2000; Igarashi et al., 2001; Alexander and Morris, 2006). This shows that these two different methods are complementary.

Functional network of identified 14-3-3 interactors

From the barley 14-3-3 interacting proteins that we have identified here, three interesting cellular processes can be highlighted. First; the Calvin cycle, according to the text books, consists of 10 main enzymes, six of which have been identified as published 14-3-3 targets (Figure 6A). In addition to these 6 Calvin cycle enzymes, another novel 14-3-3 target was identified that does not belong to the Calvin cycle but is necessary for the Calvin cycle, viz. carbonic anhydrase. This enzyme plays an important role in the conversion of carbon dioxide into carbonic acid (Werdan and Heldt, 1972), which is then converted into glyceraldehyde-3-phosphate (GAP) by the Calvin cycle. The second process emerging from the 14-3-3 target analysis that is densely populated with 14-3-3 targets is situated in sugar metabolism, namely glycolysis.

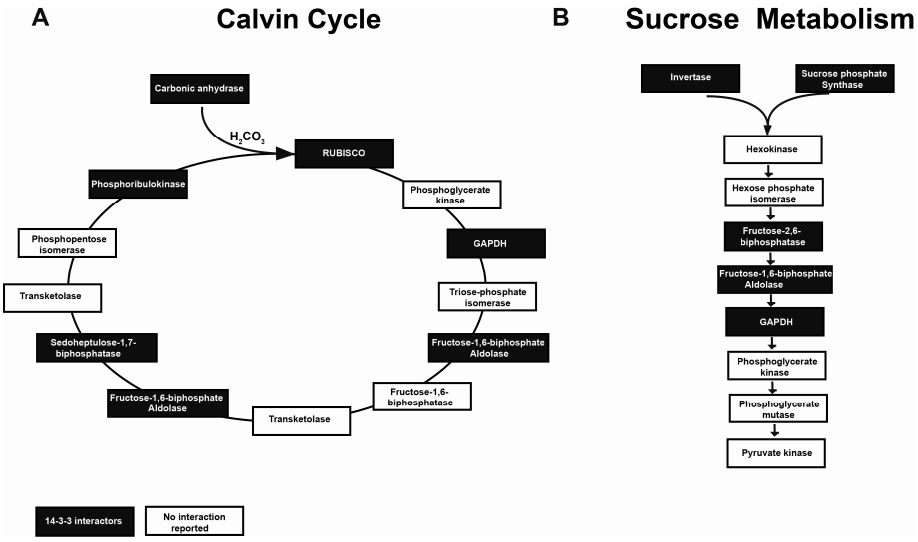

Figure 6. Calvin cycle and glycolysis are densely populated with 14-3-3 interactors

Schemes of the Calvin cycle **(A)** and the glycolysis **(B)** where 14-3-3 interactors are in black boxes and white text, show that both pathways are densely populated with 14-3-3 interactors.

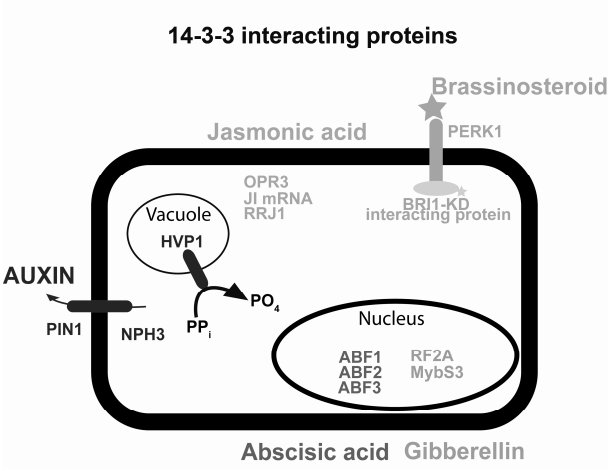

Figure 7. 14-3-3 interactors that play a role in the hormonal signal transduction.

A schematic representation of the cell showing the novel identified 14-3-3 interactors that play a role in plant hormone signal transduction. The five hormones together with the signal mediators that were identified in this study as putative 14-3-3 targets are depicted in different colors.

As shown in Figure 6B, the two enzymes that can breakdown sucrose into glucose and fructose are 14-3-3 targets: invertase and sucrose synthase. Furthermore, previously identified proteins together with proteins identified in this study show that more 14-3-3 targets are present downstream of sucrose hydrolysis, like fructose-2,6-biphosphatase (Fru-2,6-P2), fructose-1,6-biphosphate aldolase and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) (Pozuelo Rubio et al., 2003). In line
with a major function for 14-3-3 in animal cells (Darling et al., 2005) it becomes clear from the analysis of our yeast two-hybrid screen, that also plant 14-3-3 proteins are important regulators of hormonal signaling intermediates (Table 1 & 2, Figure 7). It is noteworthy that there are interesting relationships between these 14-3-3 protein targets. For example, PERK1 (BAK1) interacts with the BRI1 brassinosteroid receptor in a brassinosteroid and phosphorylation dependent manner (Wang and Chory, 2006). Interestingly, the BRI1-KD interacting protein 129 has been shown to be phosphorylated by the BRI1 receptor (Hirabayashi et al., 2004). Also the PIN1 protein (polar transport of auxin; (Wisniewska et al., 2006)) and NPH3 proteins (lateral transport of auxin; (Haga et al., 2005)) both play a key role in auxin transport. Moreover, three members of the ABF transcription factor family, transcriptional regulators of abscisic acid-responsive gene expression (Choi et al., 2000) interact with the 14-3-3 protein family.

In conclusion, our screens have revealed many established and novel putative 14-3-3 interacting proteins. The two methods employed in this study are complementary in the sense that different classes of proteins prevail in one or the other screen. In this study, a wide array of targets with a function in signal transduction has been identified in plants. Like in animal cells, 14-3-3 proteins may form a platform for cross-talk in the signal transduction pathways of the different hormones. Moreover, a picture is emerging that like in animal cells a saturation of 14-3-3 targets in the specific cellular processes, whether cyclic or linear in nature is observed.

The cataloging, functional characterization and networking of 14-3-3 targets will in the end allow the formation of a functional and dynamic map of the 14-3-3 interactome that will bring us closer to understand cellular functioning.

Table 1. Identified cDNA clones from the 14-3-3 yeast two-hybrid screen

The clones that showed activation of the reporter genes were selected and DNA was isolated. Subsequent sequences were BLAST searched against the TIGR barley EST (Tigr 9.0) database.

Names of homologues proteins	***TC # (* denotes NCBI accession #)***	***Spot #***
Carbohydrate metabolism		
Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Small Subunit (Wheat)	131295	3
Neutral Invertase (*Arabidopsis)*	137088	43
Cytosolic 6-Phosphogluconate Dehydrogenase (Rice)	146849	84
Dihydrolipoamide S-Acetyltransferase (*Arabidopsis)*	148282	107
Oxidative stress		
Manganese Superoxide Dismutase (Wheat)	138696	5
GCN5-related N-acetyltransferase (Rice)	139926	10
Glutathione S-Transferase (Rice)	151451	70
Cytosolic Glutathione Reductase (Wheat)	131783	132
Signaling		
BRI1-KD Interacting Protein 129 (Rice)	132082	12
Hv14-3-3B (Barley)	132090	15
Putative bZIP transcription factor ABI5 (Arabidopsis)	144090	13
ABA Response Element Binding Factor (Wheat)	141259	20
ABA Responsive Element Binding Factor (Arabidopsis)	134245	49
Phytoene Desaturase (Barley)	139909	26
Mybst1 (Rice)	142444	21
Serpin (Barley)	151425	22
WPK4 Protein Kinase (Wheat)	CA019527*	28
Vacuolar Proton-Inorganic Pyrophosphatase (Barley)	131805	11
Adr11 (Auxin down regulated gene 11) (Soybean)	147470	27
Putative Stearoyl-Acyl-Carrier Protein Desaturase (Rice)	139480	76
Rf2a (Rice)	152053	29
Mybs3 (Rice)	140561	111
Gibberellin-Stimulated Transcript 1 Like Protein (Rice)	148126	97
RRJ1 (Jasmonic acid responsive) (Rice)	140455	82
WERBP-1 Protein (MYB-like DNA binding domain) (Tobacco)	140267	118
Adenine Phosphoribosyltransferase (Barley)	139680	62
Orf2 (Sh1orf2)	BQ463442*	32
Helix-Loop-Helix Protein (Rice)	148621	39
Xyloglucan Endotransglycosylase (Barley)	139640	46
Putative NPH3 Protein (Rice)	136796	48
GT-1 (*Arabidopsis*)	CB883300*	60

Katanin (Rice)	140752	59
Jasmonate-Induced mRNA (Barley)	131671	66
Phospholipase D Delta Isoform 1b-Like Protein (Rice)	143006	116
PIR (Gtpase Activating-Like-Protein) (*Arabidopsis*)	140036	38
GTP Binding Protein (GTP1) (Rice)	146421	37
RING-H2 Zinc Protein	BQ460881*	67
Putative Non-Phototropic Hypocotyl 3 (NPH3)	BE421015*	65
PHD Finger Transcription Factor-Like (Rice)	135529	51
Putative Golgi-Localized Protein (Rice)	BU993045*	52
PHD Finger Transcription Factor-Like	XM476784*	75
Glycosyl Hydrolase Family (Rice)	146693	36
Mpv17 Transgene-Like (*Arabidopsis*)	140218	77
Oswrky47 (Rice)	152887	78
Putative Lipase Class 3 Family (Rice)	152325	24
Zeta1-COP (Rice)	134145	81
Similar To Adenylate Cyclase (Trypanosoma)	140534	71
Similar To Putative Receptor Protein Kinase PERK1 (Rice)	BE519502*	72
Putative AP2/EREBP Domain Transcription Factor (*Arabidopsis*)	145625	44
Probable Auxin Transport Protein (PIN1) (*Arabidopsis*)	BG309386*	93
Putative Guanylate Kinase (*Arabidopsis*)	142364	94
Guanine Nucleotide-Binding Protein Beta Subunit-Like Protein (Rice)	130838	105
Nph3 (Hordeum)	BF617760*	127
12-Oxophytodienoate Reductase 3 (Rice)	140942	133
Putative P23 Co-Chaperone (Rice)	139577	56
Protein synthesis / DNA & RNA binding proteins		
40S Ribosomal Protein S30 like (Rice)	131072	14
Putative 60S Ribosomal Protein L31 (Rice)	130806	55
Eukayotic Translation Initiation Factor 6 (*Arabidopsis*)	139393	89
9S Ribosomal Protein	BU981956*	99
Non-Ribosomal Peptide Synthetase Modules And Related Proteins-Like (Rice)	141489	103
Elongation Factor 1-Alpha (Wheat)	146580	123
Rnase S-Like Protein (Barley)	140096	23
Homologue To Histone H3.2 Protein (Human)	131253	61
RNA Binding Protein	BI960230*	115
Chloroplast proteins		
Inositol phosphatase-like protein (Rice)	146317	7
Photosystem I Reaction Center Subunit XI, Chloroplast Precursor (Barley)	146765	19
Chlorophyll A/B Binding Protein (Barley)	138667	33
PRF Light-Harvesting (Barley)	139104	31
Thylakoid Lumenal 35.8 Kda Protein, Chloroplast Precursor (*Arabidopsis*)	141769	58

Chlorophyll A/B Binding Protein Of LHCII Type III (Barley)	146726	68
Chlorophyll A/B Binding Protein (Hvlhca1) (Barley)	138676	108
Chlorophyll A/B Binding Protein (Lhcb1-2) (Potato)	146500	219
Putative Photosystem II Core Complex Proteins Psby, Chloroplast (Rice)	130812	86
Chlorophyll A/B Binding Protein (Wheat)	146521	25
Tic62 Protein	BF267751*	83
Putative Crp1 Protein (Rice)	148944	119
Signal Recognition Particle 54 Kda Subunit (Barley)	132186	126
Ferredoxin, Chloroplast Precursors (Wheat)	146610	17
Ferredoxin-Thioredoxin Reductase Subunit A (Maize)	134446	63
Stress related proteins		
Putative Aldehyde Dehydrogenase (Rice)	BI953625*	50
Fatty Acid Hydroperoxide Lyase (Barley)	141847	100
Chitin-Binding Lectin 1 Precursor (Chlamydomonas)	137023	95
Carboxymethylenebutenolidase-Like Protein (*Arabidopsis*)	139656	64
Cell division & Apoptosis		
Pelota (Rice)	134531	104
Cytokinesis 2 Protein (Yeast)	149830	47
P14arf Protein	CA018141*	114
Annexin (Rice)	134070	131
Proteasome related proteins		
26S Proteasome Regulatory Particle Non-Atpase Subunit8 (Rice)	147090	85
F-Box Family Protein-Like (Rice)	145485	45
Ubiquitin-Conjugating Enzyme E2-17 Kda (wheat)	139190	110
26S Protease Regulatory Subunit 7 (Rice)	131810	2
Proteolysis		
Degp2 Protease (*Arabidopsis*)	149647	124
ATP-Dependent Clp Protease (Rice)	139897	34
ATPase		
Plasma Membrane H^+-Atpase (Wheat)	139045	54
Atpase Like Protein (Sorghum)	150845	18
Cell wall associated proteins		
Putative Protein (Rice)	132497	16
Extensin (Volvox Carteri)	BG365257*	79
Pherophorin-Dz1 Protein	BI948976*	74
Putative beta-1,3-glucanase (Rice)	148136	106
3-Glucanase	BF625748*	102

Beta-1,3-Glucanase (Rice)	152166	69
Putative Extensin (Rice)	135984	91
Vegetative Cell Wall Protein Gp1 Precursor	AV834747*	109
Extensin-Like Protein Precursor (Maize)	141562	117
Hydroxyproline-Rich Glycoprotein DZ-HRGP Precursor (Rice)	148615	120
Storage proteins		
Putative Bark Storage Protein (Rice)	140155	96
Unknowns		
Unknown Protein	BU997476*	6
Unknown Protein	CA031470*	8
Similar To GP|20160854 P0677H08.8	BQ463267*	9
Putative Protein	139132	35
Putative Protein	141575	41
Putative Protein	148192	30
Putative Protein	139730	128
Putative Protein	134288	40
Unknown Protein	151201	53
Hypothetical Protein	151986	57
Mucin-Like Protein (*Arabidopsis*)	151069	73
No Name	148644	101
Hypothetical Protein P0461A06.20	146050	87
No Name	BG300785*	121
No Name	146647	90
Unknown Protein	134959	92
Unknown Protein	134162	98
No Name	AU090225*	113
No Name	CA017615*	80
Osjnba0086b14.2 Protein (DUF341 domain)	138807	112
No Name	BF260149*	4
No Name	BU999220*	88
Integral Membrane Protein (Unidentified)	BQ460992*	122
Similar To Arabidopsis Thaliana Chromosome II BAC T3F17; Unknown Protein	146432	125
Putative Protein	CD663282*	42

Table 2. Protein identification of the R18 eluate analyzed by LC-MS/MS.

Mass spectral data were searched against three plant databases. Accession numbers are presented for different databases where the superscript letters denotes: A: NCBI-Plants, B: OSGI, and C: ZMGI. Predicted molecular masses correlate with the gel slices from which the proteins were extracted.

	Protein name	Accesion number	Mw (kDa)	Mowse score (Peptides matched MS/MS)	Gel ID	Function	Reported	14-3-3 motif
1	Acetyl-coenzyme A carboxylase	gi:2827150[A]	254.956	110 (2)	1	multifunction	No	KEGTSAP
2	Glycine dehydrogenase	9629.m04981[B]	113.767	320 (7)	1	N-metabolism	No	KPSDTFP
3	Elongation factor 2, putative	11972.m08296[B]	94.015	57 (2)	1	Translation	No	RLAKSDP
4	Cytosolic heat shock protein 90	gi:32765549[A]	80.413	129 (2)	1	Chaperone	Yes (human)	KHFSV/ KNDKSV
5	Beta-fructofuranosidase	gi:79326306[A]	65.032	1138 (17)	1	Glycolysis	Yes	RRSNSW
6	Neutral invertase like	gi:18395144[A]	63.446	581 (8)	1	Glycolysis	Yes	KRSASWT
7	Ulp1 protease family, C-terminal catalytic domain containing protein	11981.m05812[B]	108.167	46 (2)	2	Proteolysis	No	RDHFSV/ KRSTV
8	TCP-1/cpn60 chaperonin family	9634.m00140[B]	64.081	349 (5)	2	Chaperone	No	RRALSYP
9	Catalase-1	gi:3929924[A]	56.171	52 (2)	2	Antioxidant	No	RRGSSP
10	Rubisco large subunit	gi:31087879[A]	53.141	1827 (32)	2	Calvin cycle	No	-
11	Elongation factor 1 alpha	TC299030[C]	49.137	76 (2)	2	Translation	No	KMDATTP/ KMIPTKP
12	Transferase family	9632.m05549[B]	47.971	60 (2)	2	Unknown	No	-
13	Glutamine synthetase, catalytic domain	9632.m05496[B]	47.407	73 (2)	2	N-metabolism	Yes	KHSSV/ KLWSSV/ RRPASNM
14	Phosphoribulokinase	gi:21839[A]	45.032	388 (6)	2	Calvin cycle	No	KLTCSYP
15	Sedoheptulose-1,7-bisphosphatase	gi:14265[A]	42.057	202 (4)	3	Calvin cycle	No	KVISV
16	Fructose 1,6-bisphosphate aldolase precursor	gi:8272480[A]	41.919	93 (2)	3	Glycolysis	No	RTVVSIP/ RIPPSVP
17	Glyceraldehyde-3-	gi:120668[A]	33.233	65 (2)	3	Glycolysis	Yes	KVIISAP

	phosphate dehydrogenase, cytosolic							
18	short-chain alcohol dehydrogenase	gi:7431022[A]	31.644	118 (3)	3	Signaling	No	-
19	14-3-3-like protein	gi:37903393[A]	28.880	312 (5)	3	Signaling	Yes	-
20	14-3-3 protein homologue	gi:22607[A]	29.262	836 (17)	3	Signaling	Yes	
21	Peroxidase, putative	9635.m04996[B]	42.112	111 (2)	4	Antioxidant	No	RRVLSHP/ KIESRP/ RVLGSYP
22	Carbonic anhydrase	gi:558499[A]	35.072	448 (9)	4	Calvin cycle	No	KLIRTTP
23	Putative acid phosphatase	gi:52346236[A]	29.603	630 (8)	4	Signaling	No	RDWKTVP/ RTFSRP
24	Brain specific protein	gi:303859[A]	29.127	114 (2)	4	Signaling	Yes	-
25	Bas1 protein	gi:861010[A]	29.090	315 (6)	4	Antioxidant	No	KSSLSSP/ RTLSSP
26	Thioredoxin peroxidase	gi:3328221[A]	28.131	325 (6)	4	Antioxidant	No	-
27	ATP-dependent Clp protease proteolytic subunit (Endopeptidase Clp)	TC299023[C]	27.951	78 (2)	4	Proteolysis	No	-
28	Ascorbate peroxidase	gi:15808779[A]	27.637	252 (5)	4	Antioxidant	Yes	KVLLTDP
29	Rubisco small subunit	gi:4090291[A]	19.512	383 (7)	5	Calvin cycle	No	-
30	Serine carboxypeptidase S10 family protein, similar to glucose acyltransferase	TC293082[C]	51.683	51 (2)	6	Proteolysis	No	RWLSGE

MATERIALS AND METHODS

Yeast two-hybrid screen

The Stratagene GAL4 two-hybrid phagemid vector pBD-GAL4 was used to prepare 14-3-3-GAL4 binding domain fusions using the BamHI and EcoRI restriction sites. The ORF from the Hv14-3-3 genes were amplified using PFU DNA polymerase and restriction sites were introduced into the primers. The constructs were checked for correct DNA sequence by performing sequence reactions. A Clontech MATCHMAKER two-hybrid cDNA library prepared from mRNA isolated from 5 to 7 day old leaf tissue from barley cv. Himalaya (Robertson, 2004) was a kind gift of Masumi Robertson (CSIRO Plant Industy, Canberra, Australia). The improved yeast strain PJ69-4A with genotype *MATa, trp-901, leu2-112, ura3-200, his3-200, gal4D, gal80D*, *GAL-ADE2, LYS::GAL1-HIS3, met::GAL7-LacZ*, which carries an extra reporter selecting for adenine auxotrophy (*ADE2*) was used for the yeast two-hybrid screens (James et al., 1996). Yeast transformations were performed using the lithium acetate protocol according to Gietz et al., (1992). Briefly, PJ69-4A yeast cells carrying the bait construct were grown up in SD minus LW broth for 16 hours at 30 °C. The cells were pelleted and resuspended in 1 liter of YAPD broth and grown for an additional 4 hours. Cells were pelleted, washed with one volume of water, pelleted again and resuspended in 100 ml 0.2 M LiAcetate/ TE. Again, cells were pelleted and resuspended in 10 ml end volume of 0.2 M LiAcetate. Aliquots of 2 ml yeast cells were divided in five 15 ml tubes and the following was added: 10 µg cDNA, 20 µg linearized salmon sperm DNA (Invitrogen), 12 ml of 40 % PEG-4000/ 0.2 M LiAcetate. Tubes were mixed and incubated at 30 °C for 45 minutes; subsequently the tubes were transferred to 42 °C for 25 minutes. Aliquots of 100 µl were spotted on SD minus LWHA plates and incubated at 30 °C for 7 days. In total, 1.7 million colony forming units were checked on interaction with the five barley 14-3-3 proteins. Double transformants were grown on selective plates (SD minus LWHA) for 7 days. Positive clones were streaked on plates lacking LW, one plate was used to test for *LacZ* assay and the other plate for storage. Clones that showed activation of both reporter genes (*ADE2* and *LacZ*) were selected and DNA was isolated according to the Stratagene's protocol. To prepare large quantities of DNA for sequencing reactions, the DNA isolated from yeast was electroporated into E.coli and DNA mini-preps were performed. To identify the positive clones, sequence reactions were performed using the pACT2 sequencing primer (5'-TGATGAAGATACCCCACCAAACC-3'). The resulting sequences of the cDNA clones were BLAST searched against the TIGR barley database.

Preparation of recombinant HIS tagged proteins

The 14-3-3 genes were cloned from cDNA using PCR and T/A cloning into the Invitrogen pGEM-T easy vector. Primers were designed on the 14-3-3 genes introducing BamHI sites at both sites and amplified 14-3-3 genes were subsequently cloned in frame into the Invitrogen pRSET-C vector. The ABF-His fusions were prepared by digestion of the pACT2-cDNA constructs isolated from the yeast two-hybrid screen using BamHI and BglII. Digested insert was ligated into BamHI digested pRSET-C vector, resulting in an N-terminal tagged in frame fusion protein. Recombinant proteins were prepared by electroporation of the appropriate DNA construct into BL21 E.coli cells. Positive BL21 cells were grown up in YT medium for 16 hours at 37 °C, then Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The culture was grown for another 8 hours and subsequently *E. coli* cells were pelleted and recombinant protein was isolated using the Amersham His-trap column (1 ml) according to the manufacturer's protocol. The quality of the recombinant proteins was checked by performing SDS-PAGE and western blotting using the Novagen T7-tag antibody. Far-western analysis was performed according to Moorhead et al., (1999).

Protein extraction

Barley plants were grown in the greenhouse for 7 days. Leaves of 7 day old seedlings were harvested (200 g) and ground in a blender using 500 ml of homogenization buffer (50 mM HEPES-KOH pH 7.5, 50 mM NaF, 5 mM NaPPi, 1mM DTT, 1mM PMSF, 1 tablet Roche protease inhibitor cocktail per 50 ml, 1 % PVPP). The homogenate was centrifuged for 20 minutes at 12,000*g* and the supernatant was filtered through 4 layers of Miracloth. ATP was added to a final concentration of 0.5 mM and subsequently proteins were fractionated by adding solid ammonium sulphate to a final concentration of 70 % (w/v). Precipitated proteins were pelleted at 12,000*g* for 20 minutes and the pellet was resuspended in 10 ml buffer C (50 mM Hepes-KOH pH 7.5, 1 µM Cantharidin). In order to remove remaining salts, the sample was dialyzed in a dialysis tube (NMWL = 3500 Da) against 1 liter of buffer C during 3 hours, which was refreshed every hour (total of 3 liters). After dialysis, the protein extract was cleared by centrifugation at 33,000*g* for 20 minutes and protein concentration was determined using the Bradford method (Takahashi et al., 1999).

14-3-3 affinity purification

14-3-3 affinity purification was performed using a modified protocol of Moorhead et al., (1999). The protein sample, collected as described above, was diluted with Buffer C to reach a protein concentration of 20 mg/ml. To 25 ml of this sample ATP, leupeptin and PMSF were added to reach a final concentration of 2 mM, 4 µg ml^{-1} and 0.5 mM, respectively, and this was incubated at 30 °C for 10 minutes. Finally, 10 mg of His-tagged recombinant 14-3-3 proteins (2 mg of each isoform) and NaCl (final concentration 150 mM) were added and the tube was mixed end-over-end for 1 hour at 4 °C. Thereafter, the sample was pumped through an Amersham His-trap column to bind the 14-3-3His proteins (and putative targets) using a flow rate of 0.5 ml min^{-1}. The column was washed extensively (flow rate 3 ml min^{-1}) with buffer C (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 50 mM Imidazol) until the protein concentration of the flow-through was lower than 5 µg protein ml^{-1}. A mock elution was performed using 1 ml of buffer C without the competing peptide. Subsequently, 1 ml of buffer C containing 0.5 mM R18 peptide was injected into the column with a flow rate of 0.5 ml min^{-1} to specifically elute interacting proteins. The synthetic R18 peptide (PHCVPRDLSWLDLEANMCLP) was purchased from Genscript Corporation. Finally, the column was 'stripped' by injecting buffer C plus 500 mM imidazol. Both the mock and the R18-elution were collected in fractions of 0.5 ml and concentrated using Millipore Amicon Ultra-15 tubes to a final volume of 200 µl.

Mass spectrometry

To identify the 14-3-3 interacting proteins, 50 µl of each of the mock, R18 elution, and the imidazol strip were run out on 1-D SDS-PAGE gel (12% acrylamide). Since no protein was detected in the mock elution using Coomassie Brilliant Blue R250 (note that BIO-RAD, Biosafe colloidal Coomassie was used for Figure 4), only the gel lane from the R18 eluate was analyzed. This lane was cut in six gel slices, which were then washed, digested with trypsin and peptides were extracted, as described in Friso et al., (2004). Peptides from the in-gel digests were resuspended in 5% formic acid and analyzed using nanoLC-ESI-MS/MS using a Q-TOF mass spectrometer (Waters) using gradients as described in Friso et al., (2004). Spectral data were extracted using Mascot distiller and searched with Mascot (Matrix Science) against NCBI nr - green plants, rice (OsGI - v4 from Tigr at http://www.tigr.org/), maize (ZmGI - v6; Tigr) and *Arabidopsis thaliana* (Tair v6 at http://www.arabidopsis.org/). Only proteins with two or more matching peptides matched each with a minimal ion score of 22 and a total protein MOWSE score higher than 50 were considered in this first identification step.

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Summary

Since the structure of DNA has been revealed, the majority of scientists have been focused on unraveling the genetic code. Using forward and reversed genetics approaches, many genes have been identified and characterized. To date, it is necessary to study the proteins that are encoded by these genes and map the networks of protein-protein interactions that are necessary for homeostasis in the cell. An important protein family, called 14-3-3 proteins, plays an important role in many diverse cellular processes. The 14-3-3 protein family is shown to inhibit or activate enzymes, form an adapter between two proteins, and guide subcellular localization by for example moving protein complexes from one compartment in the cell to another. In the research described in this thesis, we primarily focused on the role of barley 14-3-3 proteins in the two antagonistic plant hormone signal transduction pathways namely, abscisic acid (ABA) and gibberellins (GA).

The barley genome (5,000 Mbp) is approximately twelve times bigger than the rice genome (430 Mbp) and 33 times bigger than *Arabidopsis* (152 Mbp). This is one of the reasons that the barley genome has not been sequenced yet. In chapter 2, we identified two novel 14-3-3 genes, resulting in a total number of five identified barley 14-3-3 isoforms. The rice genome contains eight 14-3-3 isoforms and therefore, as expected from the evolutionary conservation, more barley 14-3-3 isoforms remain to be identified. *Arabidopsis* 14-3-3 proteins were initially identified as G-box binding factors, hence the name GF14. The 14-3-3 proteins were shown to interact with transcription factors that play a role in the abscisic acid (ABA) signal transduction pathway namely, VP1 and EmBP1. In chapter 2, we studied the role of the barley 14-3-3 proteins in the ABA signal transduction pathway by profiling protein and gene-expression levels in ABA treated and untreated embryonic roots (radicle). 14-3-3 protein and gene-expression levels are responsive to exogenously applied ABA. Moreover, we have shown that each of the five 14-3-3 isoforms are necessary for ABA activation of the ABA responsive HVA1 promoter. Four members of the ABF/AREB/ABI5 bZIP transcription factor family were shown to interact with 14-3-3 proteins in a yeast two-hybrid assay. The binding site of the 14-3-3 proteins within HvABI5 was identified by using site directed mutagenesis. Over-expression of the *HvABI5* gene together with the *VP1* results in a trans-activation of the HVA1 promoter in barley aleurone cells. Mutation of the 14-3-3 binding site of the HvABI5 proteins results in reduced activity of HvABI5 to trans-activate the HVA1 promoter in barley aleurone cells.

Interestingly, 14-3-3 proteins also have been shown to play an important role in mediating the signal of the ABA antagonizing pathway namely the signal transduction pathway of gibberellin (GA). Tobacco 14-3-3 proteins have been reported to regulate the

subcellular localization of a bZIP transcription factor called REPRESSION OF SHOOT GROWTH (RSG). It was shown that when 14-3-3 proteins bind RSG, the transcription factor is retained in the cytosol. When 14-3-3 interaction is abolished, RSG is located in the nucleus, where it promotes gene-expression of GA biosynthetic genes. In chapter 3, the same methodology was used as in chapter 2, RNA interference was used to reduce transcript levels of the 14-3-3 isoforms and subsequently the GA responsive α-amylase was followed. It was hypothesized that a reduction of the level of 14-3-3 proteins would result in nuclear localization of RSG and thus induction of GA biosynthetic genes. This would lead to GA biosynthesis and promotion of alpha-amylase promoter activity. The interaction between the barley RSG homologue (Rf2A) and 14-3-3 proteins was confirmed. Nevertheless, reduction of transcript levels of each individual *14-3-3* isoform did not result in promotion of the alpha-amylase promoter in the absence of GA. In contrast, the reduction of *14-3-3* transcripts led to a decreased alpha-amylase promoter (pAmy32b) activity in the presence of exogenously applied GA. We conclude that 14-3-3 proteins interact with RF2A but that reduction of *14-3-3* mRNA level does not result in an activation of GA biosynthesis. However, 14-3-3 proteins are necessary for the up-regulation of downstream genes by GA.

Calcium is an important second messenger in the cell, both the GA and ABA signal transduction pathways are known to be mediated by fast and transient changes in cytosolic calcium levels. The Ca^{2+} -dependent Ca^{2+} -release channel TPC1 has recently been shown to encode for the SV-channel. Loss-of-function mutation in the *Arabidopsis* TPC1 resulted in an *Arabidopsis* plant that has reduced sensitivity towards exogenously applied ABA. 14-3-3 proteins are known to regulate the activity of the SV-channel. Therefore in chapter 4, we have cloned the barley TPC1 gene and have performed yeast two-hybrid experiments to confirm that indeed 14-3-3 proteins interact with the TPC1/SV-channel. We show that the barley 14-3-3A isoform interacts with the cytosolic region of the TPC1 protein. We have performed gene expression profiling in combination with gene silencing experiments and followed the responsiveness of the ABA responsive HVA1 promoter. We were not able to detect altered ABA signaling when TPC1 targeted RNAi constructs were co-bombarded with the HVA1-GUS fusions. In conclusion, the barley TPC1 gene is relatively high expressed in leaf tissue. More research has to be done on the localization of the monocot and dicot TPC1 protein, we were not able to clearly demonstrate the localization of the HvTPC1 protein.

In chapter 2 and 3, we have shown that the barley 14-3-3 proteins play important roles in the ABA and GA signal transduction pathways. To identify 14-3-3 interacting

partners that play an important role in hormonal regulation of seed germination, we performed a large scale 14-3-3 affinity purification using protein extracts derived from germinating barley embryos. In chapter 5, we have identified 11 proteins that specifically interact with the barley 14-3-3 proteins like, RuBisCO activase, Elongation factor 1A, Invertases, and 14-3-3 proteins. None of these interacting proteins were associated with either ABA or GA signal transduction pathways. We hypothesize this is due to the inefficiency of the 14-3-3 affinity purification system. Moreover, the regulatory proteins in the cell are low abundant proteins and are therefore difficult to identify using mass spectrometry, especially using barley since the barley proteome databases are still limited.

To get a better understanding of the 14-3-3 interactome in barley, in chapter 6, we performed five individual yeast two-hybrid screens using the five barley 14-3-3 isoforms as bait. These screens resulted in the identification of hundred-and-thirty-two putative 14-3-3 interactors. Three of them, were ABA responsive element binding factors (ABFs), which are member of the ABF/AREB/ABI5 bZIP transcription factor family. These ABF bZIP transcription factors bind to, and subsequently activate, *cis*-elements in ABA responsive promoters. Further research has to reveal the molecular function of the 14-3-3 interaction with the ABF bZIP transcription factors. In parallel to the yeast two-hybrid screens, we performed a large scale 14-3-3 affinity purification using the same plant material as was used for the preparation of the yeast two-hybrid cDNA library. The 14-3-3 affinity purification resulted in 30 14-3-3 interactors, of which 10 proteins were also identified in the yeast two-hybrid screens. Many interactors identified in this chapter were characterized in previous reports like; RF2A, serpin, WPK4 and the P-type H^+-ATPase. These proteins function as proof of principle and show that the methods are complementary since there is overlap but interestingly, some of the known 14-3-3 interactors were not identified in the yeast two-hybrid screen but were identified in the 14-3-3 affinity purification and vice versa.

In conclusion, we have shown in this thesis that barley 14-3-3 proteins are necessary for ABA and GA signal transduction. The characterization of the interaction between barley 14-3-3 proteins and four members of the barley ABF/AREB/ABI5 bZIP transcription factor family corroborated the role of 14-3-3 proteins in the ABA signal transduction pathway. To get a better understanding of the role of the interactions between the 14-3-3 proteins and the ABFs, *in vivo* studies should be performed by for example complementation studies with mutant versions of the ABF proteins. We hypothesize that 14-3-3 proteins are the adapter protein that keeps transcription factor complexes together on the different *cis*-elements present in promoters of ABA responsive genes.

Samenvatting

Sinds de structuur van de DNA helix was ontdekt, is de hoogste prioriteit in de biologische wetenschappen verschoven naar het ontrafelen van deze complexe genetische code. Met behulp van 'forward en reversed genetics' zijn er in korte tijd velen genen gekarakteriseerd. Aangezien veel van de genen reeds geïdentificeerd zijn, is het nu van belang om de eiwitten en hun netwerken te bestuderen. De 14-3-3 eiwit familie is een belangrijk eiwit dat een rol speelt in verschillende processen zoals, celdeling, apoptosis, en hormoon signaal transductie. 14-3-3 dimeren vormen een adapter waarin plaats is voor twee andere eiwitten en vormen zo een stabiel complex. Zij kunnen op deze manier enzymatische activiteit (in)activeren, subcellulaire lokalisatie reguleren, of transcriptie niveaus reguleren d.m.v. bijvoorneeld transcriptie factoren in het cytosol te behouden. De primaire taak in het onderzoek beschreven in dit proefschrift was om de rol van gerst 14-3-3 eiwitten te bestuderen in de twee hormonale signaaltransductie ketens, abscisicine zuur (ABA) en gibberelline zuur (GA).

Het gerst genoom (5.000 Mbp) is ongeveer twaalf keer zo groot als het genoom van rijst (430 Mbp) en ongeveer drieëndertig keer zo groot als dat van *Arabidopsis* (152 Mbp). Dit is dan ook één van de redenen dat het genoom van gerst nog niet volledig geïdentificeerd is. De eerste 14-3-3 eiwitten geïdentificeerd in planten werden gekarakteriseerd als G-box bindings eiwitten, vandaar de naam GF14. G-boxen zijn DNA sequenties in promotoren die verantwoordelijk zijn voor de activiteit van de desbetreffende promotor. Er werd geobserveerd dat de 14-3-3 eiwitten de transcriptie factoren EmBP1 en VP1 binden en zodoende zich op de promotor van ABA geïnduceerde genen bevindt. Om een beter inzicht te krijgen van de rol van de gerst 14-3-3 eiwit familie in de ABA signaaltransductie keten, hebben wij de niveaus van de 14-3-3 mRNA en eiwit gevolgd in ABA behandelde en onbehandelde embryo wortels (radicles). 14-3-3 eiwit en genexpressie zijn beide geactiveerd in ABA behandelde radicles. Door middel van RNA interferentie werd tevens ook geobserveerd dat elk 14-3-3 isovorm een belangrijke rol speelt in de activering van de ABA induceerbare promotor, HVA1. Met behulp van de yeast two-hybrid methode werden vier kandidaat eiwitten geïdentificeerd die een interactie aangaan met de gerst 14-3-3 eiwitten. Deze vier eiwitten zjin leden van de ABF/AREB/ABI5 familie en spelen een zeer belangrijke rol in de ABA signaaltransductie keten. Potentieel zijn dit de eiwitten die 14-3-3 eiwit nodig hebben voor het doorgeven van het ABA signaal. De interactie tussen HvABI5 en 14-3-3 eiwitten werd in hoofdstuk 2 in detail bestudeerd. Er werd aangetoond dat een mutatie van de twee mogelijke 14-3-3 interactie plaatsen in de HvABI5 sequentie, de interacties tussen HvABI5 en de 14-3-3 eiwitten verbreekt. Tevens werd de *in vivo* functionaliteit van de gemuteerde versie van HvABI5 getest. De

gemuteerde HvABI5 heeft een gereduceerde activiteit, hieruit kan geconcludeerd worden dat de 14-3-3 interactie met HvABI5 van groot belang is voor het doorgeven van het ABA signaal in gerst aleurone cellen.

Naast het feit dat wij in hoofdstuk 2 hebben aangetoond dat 14-3-3 eiwitten van groot belang zijn voor het doorgeven van het ABA signaal, zijn er reeds ook al een aantal indicaties dat 14-3-3 eiwitten een belangrijke rol spelen in de antagonistische signaal keten van ABA namelijk die van GA. Het was aangetoond dat 14-3-3 eiwitten een interactie aangaan met een transcriptiefactor REPRESSION OF SHOOT GROWTH (RSG). Dit eiwit activeert de genexpressie van GA biosynthese eiwitten. 14-3-3 eiwtten binden RSG in het cytosol en zorgen ervoor dat RSG niet de kern in kan en zodoende geen genen kan activeren. In onderzoek beschreven in hoofdstuk 3 hebben we dezelfde methodologie gebruikt als in hoofdstuk 2, RNA interferentie werd uitgevoerd in combinatie met het volgen van de GA geïnduceerde promotor van α-amylase. Met het doel om te bestuderen of de reductie van 14-3-3 eiwitten in aleurone cellen, er toe zou leiden dat RSG de kern in zou gaan en vervolgens GA biosynthese genen zou aan kunnen zetten. GA biosynthese werd gemeten d.m.v. het volgen van de α-amylase promotor. De interactie tussen de gerst RSG homoloog, RF2A, werd bevestigd met behulp van de yeast two-hybrid methode. Alle vijf de isovormen gaan sterke interactie aan met RF2A. De reductie van 14-3-3 mRNA niveaus in de aleurone cellen leverde niet het verwachte resultaat. De reductie van 14-3-3 mRNA resulteerde niet in een activatie van de GA induceerbare α-amylase promotor, in tedendeel, de signaal transductie van GA naar de α-amylase promotor werd verslechterd. Hieruit werd geclouncludeerd dat 14-3-3 eiwitten, net zoals in de ABA route, een rol speelt in het doorgeven van het GA signaal.

In het onderzoek beschreven in hoodstuk 4 hebben wij het eiwit bestudeerd dat zich onlangs heeft gepresenteerd als het eiwit dat verantwoordelijk is voor de Slow Vacuolar (SV) kanaal. Calcium is een zeer belangrijk signaal molecuul in de cel, het is bekend dat de signaal routes van ABA en GA beide worden doorgegeven door snelle en hoge cytosolische calcium fluctuaties. Een onderzoek dat recent is gepubliceerd heeft aangetoond dat de TPC1 gen van *Arabidopsis* verantwoordelijke is voor de SV stroom in de vacuole. Tevens hebben dezelfde autheurs van dit onderzoek ook aangetoond dat een plant zonder het TPC1 eiwit minder goed reageerd op ABA behandeling. Dit autheurs suggereren dat het TPC1 kanaal verantwoordelijk is voor de calcium verhogingen in de cel die belangrijk zijn voor het doorgeven van het ABA signaal. Aangezien reeds bekend was dat 14-3-3 eiwitten de activiteit van het SV kanaal reguleren hebben wij in hoofdstuk 4 aangetoond dat het gerst TPC1 ook inderdaad interactie aangaat met 14-3-3 eiwitten.

Tevens hebben wij met behulp van RNA interferentie getracht om aan te tonen dat ook in gerst het TPC1 eiwit een belangrijke rol speelt in het doorgeven van het ABA signaal. In onze experimenten lijkt het erop dat het TPC1 eiwit geen belangrijke rol speelt in de activatie van genen door de ABA route.

In hoofdstuk 2 en 3 hebben wij aangetoond dat de 14-3-3 eiwitten een belangrijke rol spelen in de ABA en GA signaaltransductie routes. Hierdoor hebben wij in hoofdstuk 5 een experiment uitgevoerd om de belangrijke ABA en GA signaal mediatoren te identificeren. 14-3-3 partners in kiemende gerst embryo's werden geïdentificeerd d.m.v. 14-3-3 affiniteits chromatografie. Elf 14-3-3 partners werden geïdentificeerd waaronder, Invertase, 14-3-3 eiwitten, RuBisCO activase, Elongation factor 1A. Geen van deze eiwitten hebben een uitgesproken functie in een van de twee hormoon routes waarin wij geïnteresseerd waren. De reden dat er veel enzymen en hoog abundante eiwitten geïdentificeerd werden heeft volgens ons twee redenen. I) De efficiëntie van de 14-3-3 affiniteits chromatografie en voornamelijk de identificatie met LC-MS/MS is laag. II) Hoog abundante eiwitten winnen de competitie met de laag abundante eiwitten waardoor slechts hoog abundante eiwitten geïdentificeerd worden.

Om een beter inzicht te krijgen in de gerst 14-3-3 interactome, hebben we in hoofdstuk 6 twee verschillende methodieken gebruilkt om 14-3-3 interactie partners te identificeren. Namelijk, vijf individuele yeast two-hybrid screens werden uitgevoerd en parallel werd er in hetzelfde plant materiaal waar de cDNA library van gemaakt was, een 14-3-3 affiniteit chromatografie experiment uitgevoerd. Deze gecombineerde aanpak leverde meer dan 150 interactors op. De 14-3-3 affiniteit chromatografie resulteerde in 30 eiwitten, waarvan 10 eiwitten ook in de yeast two-hybrid screens geïdentificeerd werd. In beide methodieken werden eiwitten geïdentificeerd die reeds beschreven zijn in de literatuur als zodanig 14-3-3 partners, deze eiwitten bevestigen dat de methodieken functioneel zijn.

In conclusie, the 14-3-3 eiwitten in gerst aleurone cellen spelen een cruciale rol in het activeren van genen die van belang zijn in de twee hormonale routes die zaad kieming reguleren. In hoofdstuk 5 en 6 hebben we data gegenereerd die de resultaten in hoofdstuk 2 en 3 bevestigen. Meer onderzoek is nodig om daadwerkelijk de precieze functie van de interacties tussen de 14-3-3 eiwitten en de targets te weten te komen. Gerst is hiervoor niet het juiste modelsysteem, in *Arabidopsis* zouden mutanten opgevraagd kunnen worden die bijvoorbeeld een van de ABF genen mist. Deze planten zouden getransformeerd kunnen worden met een ABF die geen 14-3-3 bindingsplek heeft. Zo zou de *in vivo* functie van de 14-3-3 interactie met de ABFs beter bestudeerd kunnen worden.

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- Poster presentation at GRC on “The Biology of 14-3-3 proteins” 2006 (Oxford, UK)
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